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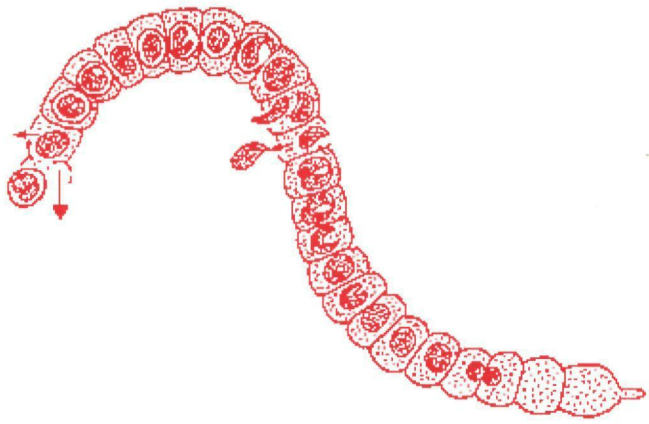

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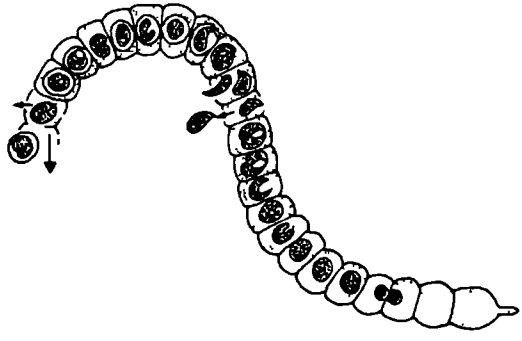

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A study of segmented filamentous bacteria in the ileum of mice

Ter nagedachtenis van mijn ouders

Aan Lisette en Sander

A study of segmented filamentous bacteria in the ileum of mice

Een wetenschappelijke proeve op het gebied van de
Medische wetenschappen, in het bijzonder de Geneeskunde

PROEFSCHRIFT

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Voorwoord

Het darmkanaal van mens en dier herbergt een letterlijk onnoemelijk aantal bacteriën, als geheel aangeduid met termen als darmflora en microflora en bestaande uit honderden bacteriële soorten. Zo kunnen in de ontlasting van de mens, die een goede weerspiegeling is van de situatie in de dikke darm, meer dan 10^{10} bacteriën per gram aangetroffen worden. Hoewel een deel van de darmflora van gezonde mensen en dieren onder bijzondere omstandigheden ziekteverwekkend (pathogeen) kan worden, wordt het grootste deel van de darmflora gevormd door apathogene bacteriën. Deze zijn van belang voor een optimale darmwerking, o.a. doordat zij de darmperistaltiek stimuleren en slecht verteerbare voedingscomponenten afbreken.

Kolonisatieresistentie (KR) van het maagdarmkanaal is het complex van darmflora-geassocieerde faktoren dat de kolonisatie van pathogene bacteriën in de darm voorkomt of beperkt. Het is onduidelijk welke bacteriële species hierbij de belangrijkste rol spelen. Wel staat vast dat KR van essentieel belang is voor de weerstand van mens en dier tegen darminfekties.

Muizen zijn de meest gebruikte proefdieren in biomedisch onderzoek. Ze worden ook frequent gebruikt voor onderzoek naar de fundamentele achtergronden van de KR van het maagdarmkanaal. De darmflora van de muis is uitgebreid bestudeerd. Ook is van een beperkt aantal bacteriesoorten het gastheerbeschermend effekt in infektieproeven getest. Dit onderzoek is uitgevoerd met bacteriesoorten die op kunstmatige voedingsbodems (*in vitro*) te kweken zijn. Een nog onbekend aantal darmbacteriën is echter moeilijk of niet kweekbaar *in vitro*. Deze bacteriën stellen onbekende eisen aan hun micromilieu, om zich te kunnen vermenigvuldigen. Een voorbeeld is een nog niet gekarakteriseerde bacterie (of groep van bacteriën) die wordt aangetroffen in het laatste deel van de dunne darm van vele diersoorten, waaronder de muis: de zogenaamde gesegmenteerde, filamenteuze (draadvormige) bacterie ('segmented, filamentous bacterium', SFB). In de literatuur wordt gespeculeerd over een mogelijke funktie van SFB's in de vorming van weerstand van de gastheer tegen bepaalde darminfekties. Op grond van zijn microscopische vorm en struktuur is de SFB wel gemakkelijk herkenbaar, maar de SFB kon niet worden gescheiden van andere bacteriën. Het niet kunnen beschikken over zuivere

SFB-cultures heeft het onderzoek naar betekenis en funktie van SFB's voor de gastheer lange tijd gestagneerd.

Dit proefschrift karakteriseert SFB's door onderzoek naar determinanten van het voorkomen van SFB's en door onderzoek naar de mogelijke interaktie tussen SFB's en gastheer. Eerst wordt een literatuuroverzicht gegeven (hoofdstuk 1.2). In hoofdstuk 1.3 wordt onderzoek beschreven naar het voorkomen van SFB-achtige microörganismen in diverse diersoorten. In de hoofdstukken 2.1 t/m 2.7 wordt de invloed van diverse gastheer- en externe faktoren op de SFB-kolonisatie *in vivo* beschreven. Het isoleren van levende SFB's uit de darm van de muis en het vervolgens produceren van een monocultuur van de bacterie met kiemvrije muizen als gastheer, vormen het onderwerp van hoofdstukken 3.1 en 3.2. In hoofdstukken 4.1 en 4.2 worden proeven beschreven waarin de invloed van SFB's op het mucosale immuunsysteem en KR werd onderzocht. De resultaten van het gehele onderzoek worden geëvalueerd en samengevat in hoofdstuk 5.

Chapter 1

General introduction

1.1 Organization of the thesis

The aim of this thesis is to characterize segmented, filamentous bacteria (SFBs) in the ileum of mice. A literature survey is given in Chapter 1.2. Chapter 1.3 describes a screening of various animal species, including human patients, for the presence of intestinal, SFB-like microbes. In Chapters 2.1 to 2.7, selected host-associated and environmental determinants of SFB colonization are described. Chapters 3.1 and 3.2 deal with techniques that enabled the production of a monoculture of SFBs in mice. In Chapters 4.1 and 4.2 effects of SFBs on the immune system and the colonization resistance (CR) of mice are described. Finally, the results described in this thesis are evaluated and summarized in Chapter 5.

1.2 Intestinal segmented, filamentous bacteria

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CONTENTS

I. SUMMARY

Segmented, filamentous bacteria (SFBs) are autochthonous, apathogenic bacteria, occurring in the ileum of mice and rats. Although the application of formal taxonomic criteria is impossible due to the lack of an *in vitro* technique to culture SFBs, microbes with a similar morphology, found in the intestine of a wide range of vertebrate and invertebrate host species, are considered to be related. SFBs are firmly attached to the epithelial cells of the distal ileal mucosa, their preferential ecological niche being the epithelium covering the Peyer's patches. Electron microscopic studies have demonstrated a considerable morphological diversity of SFBs, which may relate to different stages of a life cycle. Determinants of SFB colonization *in vivo* are host species, genotypical and phenotypical characteristics of the host, diet composition, environmental stress and antimicrobial drugs. SFBs can survive *in vitro* incubation, but do not multiply. On the basis of their apathogenic character and intimate relationship with the host, it is suggested that SFBs contribute to development and/or maintenance of host resistance to entero-pathogens.

II. INTRODUCTION

There are many species of bacteria that form filaments (1). Here we describe segmented, filamentous bacteria (SFBs) which form a group of bacteria with similar morphology and identified on the basis of their morphology only. Any gliding movement, which is a characteristic of many filamentous bacteria (1), has not been reported for SFBs.

SFBs are part of the gastrointestinal (g.i.) microbiota in various animal species. SFBs from mice and rats have been most widely studied. However, 81 years before the first report of SFBs in mice in 1962 (2), SFB-like microorganisms in the intestine of insects were already described by Leidy (3). Despite their early discovery, SFBs have not been extensively studied. This probably relates to the fact that attempts to culture SFBs *in vitro* have been unsuccessful up until now (4-13). As a consequence, SFBs can only be studied *in vivo*, which apart from being laborious, excludes metabolic processes in SFBs to be described. Here, we review

the morphological properties of SFBs, their habitat and occurrence in various host species. A life cycle for SFBs is proposed and determinants of SFB colonization are discussed. Finally, we hypothesize that SFBs increase host resistance to enteropathogens.

III. CHARACTERISTICS OF SFBs

III.1. Habitat and attachment

Because SFBs cannot yet be cultured *in vitro*, their presence in the intestine has not been quantified by common microbiological techniques. Scanning electron microscopy indicates that the highest density of SFBs is in the distal ileum of mice and rats (14). SFBs are absent in suckling mice and rats, but appear after weaning (15, 16). Martin and Holland (17) found large numbers of SFBs in the anterior region of the ileum of *Hymenolepis*-infected rats. Apparently, *Hymenolepis* infection alters the habitat of SFBs.

Within the ileum, SFBs are attached either to the epithelium of intestinal villi or to that of Peyer's patches (18, 19). Villus-associated SFBs are most abundant on the lateral and apical third of the villi (14; Fig. 1). SFB populations are most dense when associated with the epithelium covering the lymphoid tissue of the Peyer's patches in mice and rats (13, 18, 19; Fig. 2). In domestic fowl, Glick *et al.* (20) and also Käufer and Sobiraj (8) observed dense SFB populations attached to the villi of the caecal tonsil (a lymphoid aggregation in the caecal mucosa), whereas these bacteria were absent in the other parts of the caecum. Apparently, SFBs have a preference for attachment to lymphoid tissue. This might relate to growth promoting substances secreted by the lymphoid tissue (21) or to the absence of goblet cells (specialized, mucin-producing epithelial cells) in Peyer's patch epithelium (19). Scanning and transmission electron microscopy have shown that SFBs induce deep indentations at the luminal side of the host epithelial cells ("cup-shaped depressions" or "nipple-like extensions"), but do not penetrate the epithelial plasma membrane nor induce their phagocytosis by the host cells (5, 11, 16, 22, 23; Fig. 3). Neither SFBs themselves nor acute inflammatory reactions have been found in the lamina propria, i.e. the layer of loose connective tissue just below the

monolayer of epithelial cells (5, 23). SFBs merely cause displacement, absence or modification of microvilli in the vicinity of the attachment (5, 11, 16; Fig. 3). The attachment site is characterized by the presence of dense aggregates of host cell submembrane cytoplasmic filaments (16, 22, 23; Fig. 3). These actin-like microfibrils may mediate a sol-gel transformation of the apical cytoplasm of the host cell and thus stabilize the attachment pocket (11).

To visualize the epithelial attachment site of SFBs, the mucous layer covering the epithelium and the attached bacteria has to be removed (24). Davis *et al.* (25, 26) showed in rats and dogs that the majority of attached SFBs is separated from the gut lumen by a thin film of material which is probably mucus. Rozee *et al.* (27) confirmed this using methods that do not remove the mucous blanket from the mucosal surface.

Thus, SFBs are firmly attached to the epithelial cells of the distal ileal mucosa. Their preferential sites are the cells covering the Peyer's patches. SFBs are separated from the gut lumen by the mucous layer that covers the mucosal surface.

III.2. Morphology and proposed life cycle

Electron microscopic studies have demonstrated a considerable morphological diversity of SFBs. Based on these studies, we propose the SFB life cycle shown in Figure 4. The available morphological data are described below.

With the use of scanning electron microscopy (4, 22) and phase contrast microscopy (28) two different morphotypes of SFB filaments can be distinguished in rats and mice. Length of the two morphotypes ranges from 2 μm to more than 1000 μm (own unpublished observations). One morphotype has a diameter of about 1.3 μm, has defined transverse septa and shows distinct rounded single cells which cause a beaded appearance (Fig. 4, Stage X). Sometimes, this morphotype has a terminal spherical body with a diameter of about 1.6 μm. The other morphotype has a diameter of about 0.8 μm and shows no clearly externally visible segmentation (Fig. 4, Stages II-IV). Both types of filaments have smoothly rounded free ends. Shorter forms of the beaded filaments may have rough, irregular free ends (18). These two morphotypes are shown in Figure 5, which is a light micrograph of a Gram-stained mucosal smear from the ileum of a mouse.

A third form of SFBs is the so-called holdfast segment (Fig. 4, Stage I). It is

Fig. 4

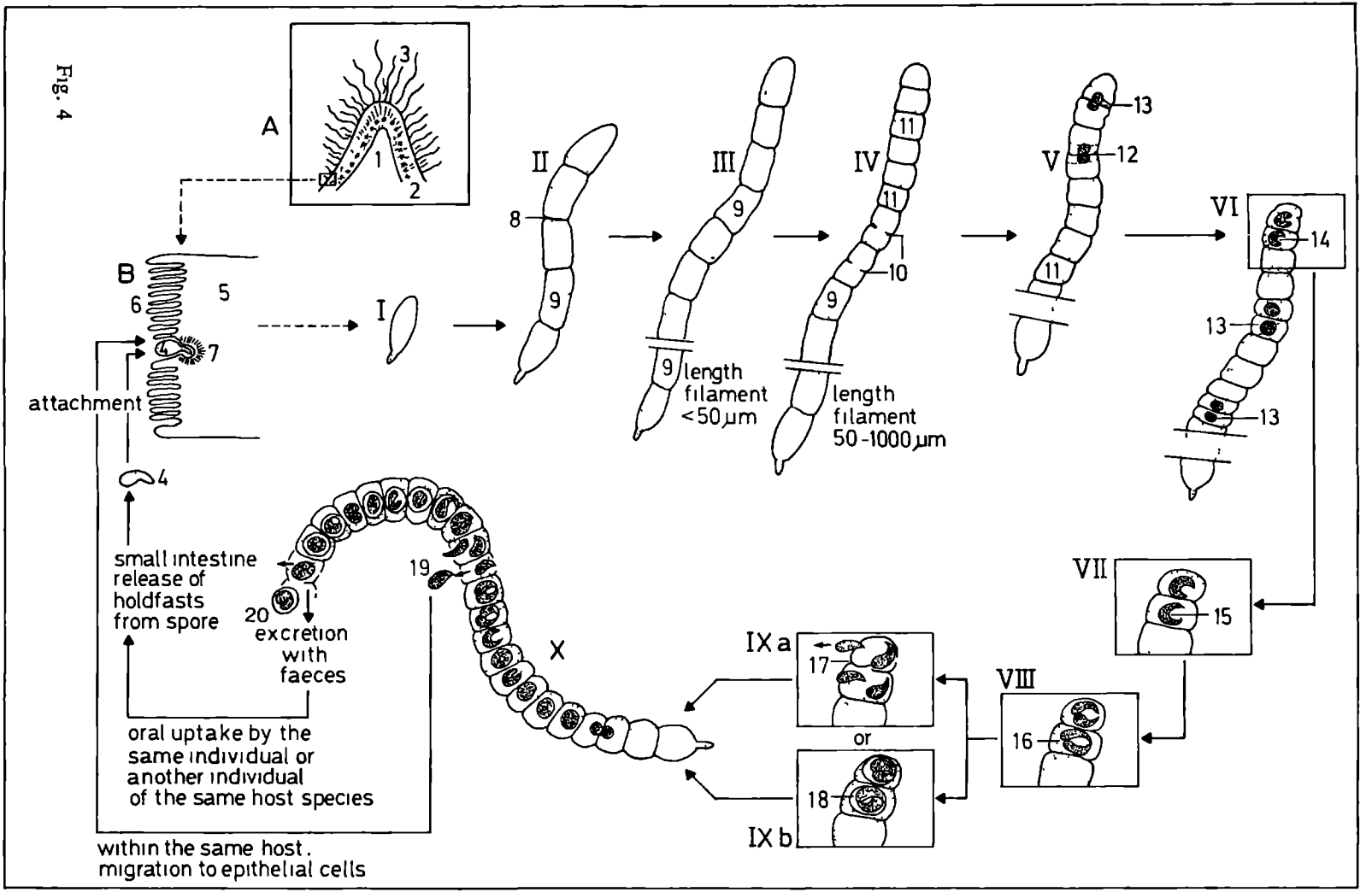
A
1
2
3
B
4
5
6
7
attachment
I
II
8
9
III
9
length filament <50 µm
IV
9
10
11
length filament 50-1000 µm
V
11
12
13
VI
13
14
13
VII
15
VIII
16
IX a
17
or
IX b
18
X
19
20
excretion with faeces
small intestine release of holdfasts from spore
oral uptake by the same individual or another individual of the same host species
within the same host. migration to epithelial cells

Fig. 4. Schematic presentation of the proposed life cycle of segmented, filamentous bacteria (SFBs). (A) Small intestinal villus with SFBs adhering to its epithelium. (B) Site of attachment of one SFB holdfast segment after magnification of a transverse section of the epithelial layer. (I) Holdfast segment. (I-X) Successive developmental stages of SFB filaments. The arrows from stage I to IXa and IXb indicate the conversion of a stage into the next. Stage X is in fact a combination of stages V-VIII, IXa and IXb in one filament. (1) Lamina propria. (2) Epithelium. (3) SFBs. (4) Individual holdfast segment. (5) Apical cytoplasm of epithelial cell. (6) Microvilli. (7) Electron dense area around nipple-like appendage of holdfast segment. (8) Transverse septum. (9) Longer, undifferentiated segment. (10) Formation of transverse septa. (11) Shorter, undifferentiated segment. (12) Septum between two reproductive segments. (13) Spherical, forespore-like, intracellular body (ICB) (14) C-shaped ICB with rounded ends. (15) C-shaped ICB with pointed ends. (16) Pair of holdfasts. (17) Disintegration of transverse septa and lateral cell walls. (18) Spore containing two holdfasts. (19) Released holdfast. (20) Released spore.

attached to an epithelial cell of the ileal mucosa (Fig. 4, Section B) as described above. The form of the holdfast segment varies from bean- to teardrop- or bulb-shaped with a diameter of about 1.6 μm (4). The holdfast segment as well as the two filament morphotypes have been demonstrated in the ileum of both mice and rats (4).

Transmission electron microscopy has revealed the internal ultrastructural details of the two filament forms and the holdfast segment. They possess the ultrastructure of a procaryotic microorganism as they do not contain a discrete nucleus: the holdfast segments have a compact and the filaments a dispersed nucleoid (5, 22). Furthermore, they have a gram-positive cell wall (11, 22). The filaments can have segments without or with intracellular bodies (ICBs) (5, 11, 22).

Shorter filaments have undifferentiated segments with length of 2-3.4 μm (Fig. 4, Stages II and III). The segments are separated by transverse septa. The free ends of long filaments (length, 50 to more than 1000 μm) have segments (length of 1-1.7 μm), which may be formed by a division of the longer, undifferentiated segments (Fig. 4, Stage IV). These segments in turn can partition into two compartments of unequal length. In the small compartments spherical ICBs develop (Fig. 4, Stage V) which are successively transformed into C-shaped ICBs (Fig. 4, Stages VI and VII). The C-shaped ICBs may be direct precursors of holdfasts (Fig. 4, Stage VIII), because their shape and compact nucleoid are very similar to that of attached holdfasts. After disintegration of the compartment, the holdfasts are released (Fig. 4, Stage IXa). Newly formed holdfasts may also be incorporated into spore-like structures (Fig. 4, Stage IXb). The forming of holdfasts and spores containing two holdfasts starts in the terminal segments. The deposition of spore walls is morphologically identical to that in *Clostridium* and *Bacillus* (22).

The spores probably ensure the transmission of SFBs from one individual to another. Thus after oral ingestion by a host individual, the spores release holdfasts. Transmittable forms of SFBs have been described by Koopman *et al.* (29). They are more sensitive to heating than spores from *Bacillus* and *Clostridium* (29). Resistant forms of SFBs must have induced the appearance of SFBs in germ-free mice housed in separate cages, but in the same isolator as SFB-harbouring mice (30). Thus, neither direct contact between animals nor ingestion of freshly excreted faeces are necessary for transmission. SFB colonization is not inhibited in mice which are prevented from coprophagy (31), which implies that the SFB life cycle in one animal is maintained by direct attachment of released holdfasts (Fig. 4, Stage X/19). The transmission of SFBs from one animal to another only occurs within the same species. Germ-free mice and rats are colonized by SFBs after oral administration of intestinal homogenate derived from mice and rats, respectively, but not vice versa (13, 32).

There are thick and beaded, as well as slender and straight filaments in mice and rats. This has raised the question whether these represent two different developmental stages or two (sub)species of SFBs (4, 33). Filaments without clearly visible external segmentation are probably transformed into filaments with a beaded appearance (22). This would indicate that they represent two developmental stages of SFBs. The same two morphotypes occur in the caecum of the domestic fowl (8). Many of the internal and external ultrastructural details of murine SFBs have also been observed in the filamentous *Arthromitus*-like bacteria in the termite intestine (34, 35). These include filaments ("trichomes") with or without externally visible segmentation, attachment of filaments to the intestinal wall and formation of spores in the free ends of the filaments (34, 35). These spores are able to survive outside the host (34, 35). Perhaps, these bacteria in termites have a life cycle similar to that of SFBs in rodents.

III.3. Subcolonization by epibionts

There can be subcolonization of SFB filaments by bacteria of variable size and form. As early as 1881, Leidy (34) described so-called epibionts adhering to filamentous bacteria in the intestine of termites. These epibionts vary from small rods or coccoids to taller, elongated or fusiform-shaped bacteria. Subcolonization

of SFBs from mice is illustrated in Figures 6 and 7. Figure 6 is a scanning electron micrograph of the ileum of a mouse and Figure 7 is a light micrograph of a Gram-stained mucosal smear from a mouse ileum. Käufer and Sobiraj (8) first reported epibionts on SFBs in vertebrate hosts. In the proximal caecum of chickens, they observed large numbers of small, rod-shaped bacteria that subcolonized SFBs, often along their entire length. Blumershine and Savage (4) showed in mice curved, rod-like structures in very close contact with an attached holdfast, which possibly was a single colonizing epibiont. Koopman *et al.* (16) demonstrated subcolonization of SFBs by numerous small, rod-shaped bacteria in 20 day-old mice.

In the chicken caecum as well as in the mouse ileum, the interaction between SFBs and epibionts involves a layer of fuzzy material, probably pili or fimbriae, on the outer surface of the two microbes (8, 16). Koopman *et al.* (16) speculated that the small rods were lactobacilli and that their adherence to SFBs involved 'fuzzy layers' on the outer surface of the SFBs. Margulis *et al.* (35) studied gut wall-associated, filamentous, segmented, spore-forming bacteria in the hindgut of wood-eating insects and frequently observed rod-shaped epibionts surrounding SFBs, especially at their distal ends. The layer between SFBs and epibionts had a fuzzy, loose nature (35).

The significance of the epibiotic symbiosis of SFBs and rod-shaped bacteria is unknown. It seems that subcolonization of SFBs in mice occurs more frequently in fasted than fed animals (own unpublished observations).

III.4. Host species

Table 1 summarizes the host species in which SFB-like bacteria have been detected. Little is known about the taxonomic relationship between these microbes, but their striking morphological resemblance could lie in the possibility that they represent one taxonomic group of bacteria. The first report on intestinal, spore-forming, filamentous, sometimes segmented ('articulate'), bacteria is by Leidy (34). He found these bacteria, which he referred to as plants, in the intestine of certain myriapods and termites and gave them the genus name *Arthromitus* (34, 36). It took more than a century before Margulis *et al.* (35) detailed the symbiotic status and ultrastructure of arthromitids in the hindgut of termites. The genus *Arthromitus* has initially been placed in the order Caryophanales, fam.

Arthromitaceae (36). Later, in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (37), they fell under Part 15: endospore-forming rods and cocci, fam. I: Bacillaceae, and in *Bergey's Manual of Systematic Bacteriology* (38) they were placed under "Genera Incertae Sedis".

After the report of Leidy (34), intestinal, filamentous, segmented bacteria were demonstrated in termites (39), cockroaches (36, 40) and frog and toad tadpoles (36). The domestic duck is the first avian species for which a related bacterium (*Anisomitus denisi*) was described (41). In addition to their morphology, the bacteria of the genera *Arthromitus* and *Anisomitus* have in common that one end of the filament is attached to the intestinal wall (37).

In the anal and vulvar pores of helminths from the hindgut of zebras, segmented, filamentous microbes were detected (42-44). It was concluded that these helminth-associated bacteria were autochthonous, intestinal microbes of the zebra. Davis *et al.* (26) and Hoskins *et al.* (45) described SFBs that were attached to the distal ileal mucosa of dogs and resembled murine SFBs perfectly. The same applies for segmented, filamentous microorganisms in cats and sheep (46). In an oral eosinophilic granuloma of a cat, filamentous bacteria were demonstrated in an area without inflammatory cells (47). These bacteria were Gram-variable, had a diameter of more than 1 μm, and some filaments had a beaded appearance. The significance of this rare finding is unclear, but the morphological resemblance of the bacteria with murine SFBs strongly suggests that they can be considered a related mammalian SFB. This is supported by two observations of SFBs in Gram-stained smears from the tonsillar mucosa of beagle dogs (own unpublished observations).

Margulis *et al.* (35) noted that similar intestinal, segmented, filamentous bacteria occur in unrelated animals such as termites, beetles, myriapods, isopods, mice, rats, guinea pigs, domestic ducks and domestic fowl. Table 1 extends this list of host species. Margulis *et al.* (35) stated that it should be possible to identify and classify large bacteria such as *Arthromitus* species on the basis of host range, habitat and microscopic morphology. More conclusively, however, would be the DNA sequencing of the SFB-like bacteria in the various host species.

Table 1. Animal species and intestinal regions in which SFB-like bacteria have been detected

VERTEBRATES			INVERTEBRATES		
Animal species	Intestinal region	References	Animal group	Intestinal region	References
Mouse	Forestomach	101.	Myriapods	Small intestine	36.
	Ileum	2,4,5,6 18,19,23, 33,59,101.		Large intestine	36.
				Rectum[2]	36.
Rat	Forestomach	58.			
	Ileum	5,6,7,10 11,14,22, 25,102.	Termites	Small intestine	34.
				Large intestine	34,35, 36,104, 105.
Guinea pig	Caecum	35.			
Dog	Ileum	26,45.			
Cat	Ileum	46.			
	Oral cavity	47.	Beetles	Intestine[1]	34,35.
Sheep	Ileum	46.			
Zebra	Large intestine	42,43,44.			
			Isopods	Intestine[1]	35.
Vervet monkey	Ileum	103.			
Chicken	Ileum	49,50,72.			
	Caecum	8,20.			
			Cockroaches	Large intestine	34,36, 40.
Domestic duck	Small intestine	41.			
	Caecum	41.			
Frog (tadpole)	Rectum	36.			
Toad (tadpole)	Intestine[1]	36.			

[1] Region not mentioned; [2] Found in lumen.

III.5. Determinants of SFB colonization within species

Attempts to identify determinants of SFB colonization within a given species have been severely hampered by the considerable inter-individual variation in SFB density. Garland *et al.* (7, 15) showed this for rats and Koopman *et al.* (48) for mice. Figure 8 gives an example of inter-individual variation of SFB colonization of the ileum of mice. There are also large variations in group mean SFB colonization values from one experiment to another. Figure 9 illustrates this. It is clear that SFBs can be undetectable in one experiment and abundant in another while mouse strain and experimental conditions such as diet, temperature, bedding, etc. are identical. Despite the considerable intangible variation in SFB colonization, a number of determinants could be identified. Some of these may have direct effects on SFBs. Others may influence SFB colonization indirectly, by changing the physiology of the host.

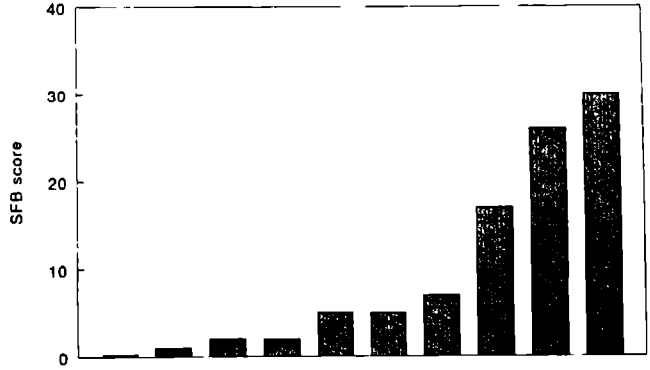

Fig. 8. Colonization by segmented, filamentous bacteria (SFBs) of the ileum of ten, apparently identical, individual mice within one experiment. SFB colonization is expressed as SFB score: percentage of SFB-positive fields, determined by light microscopic examination of Gram-stained mucosal smears of the small intestine (100 fields per smear examined at magnification of 1000x). Experimental details can be found elsewhere (55).

Autochthonous SFBs have been demonstrated in a wide range of host species. However, these bacteria may be host-specific as has been shown for mice and rats (13, 32). Thus the host species is an important determinant of SFB colonization. Localization of SFBs is also host-dependent as they have been demonstrated in the ileum of mice and rats (5, 14, 15, 22) whereas chicken SFBs occur in the ileum as well as in the caecum (8, 20, 49, 50).

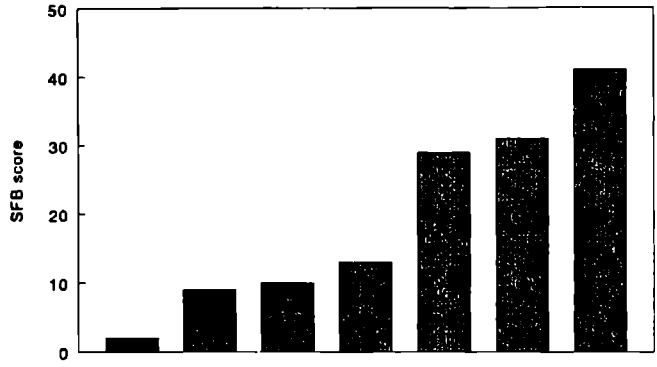

Fig. 9. Group mean colonization by segmented, filamentous bacteria (SFBs) of the ileum of seven, apparently identical groups of mice in different experiments. The groups consisted of six to ten mice. SFB colonization is expressed as SFB score (see legend to Fig. 8). Experimental details can be found elsewhere (55, 56; Klaasen *et al.*, submitted for publication).

Genotypical and phenotypical characteristics of the host determine colonization density of SFBs within one host species. Different strains of mice and rats can differ markedly in the density of SFB populations (5, 29, 51, 52). Davis and Savage (5) described that in a particular strain of mice females have more SFBs than males, but the opposite was seen in another strain. Age has been shown to be an important determinant in mice (5, 16, 51), rats (10, 15) and chickens (8, 20). The microbiological state of the host's intestine influences SFB colonization. This is demonstrated by the presence of SFBs at the caecal mucosa of mice harbouring SFBs only (53), which has never been found in conventional or specific pathogen-free mice (own unpublished observations). The immunological state of the animal may affect SFB appearance as the number of SFBs was lower in athymic mice than in their heterozygous (normal) littermates (54).

The feeding regimen and the composition of the diet are determinants of SFB colonization. In mice diets with different composition induced significant differences in SFB population density (29, 48, 55-58; own unpublished observations), but it is unknown which dietary substances are responsible for this. Fasting markedly reduces the incidence of SFBs (59).

Environmental stress clearly affects SFBs. Hyperbaric stress induces a dramatic decrease in numbers of SFBs in mice, which may relate to a general host response to this form of stress (3). Tannock (60) noted that anorexia (lack or absence of

appetite) may be associated with hyperbaric conditions. Other stress factors that tend to decrease numbers of SFBs in mice are overcrowding, lack of bedding, continuous light and housing at high temperature (61). Gamma radiation stress at sublethal and lethal doses, respectively temporarily and permanently reduces SFB populations in the rat ileum (62). These effects may relate to anorexia and/or reduction of gastrointestinal peristalsis (62). Normally attached SFBs were observed in mice treated with nitrogen mustard (63). Social stress adversely affects SFB populations in the ileum of mice (51). In domestic fowl, caecal SFBs are more abundant in litter- than in battery-reared animals (20).

Table 2. Simplified pattern of sensitivity to antimicrobial drugs of SFBs, compared to that of facultatively anaerobic and obligately anaerobic bacteria

Antimicrobial drug	Sensitivity of:				Degree of sensitivity of SFBs[2]
	Facultative anaerobes[1]		Obligate anaerobes[1]		
	Gram +	Gram -	Gram +	Gram -	
Amoxicillin	+	v	+	-	+++
Doxycyclin	+	+	+	-	+++
Gentamicin	v	+	-	-	++
Vancomycin	+	-	+	-	++
Ciprofloxacin	+	+	-	-	+++
Trimethoprim	+	v	-	-	+
Metronidazol	-	-	+	+	+
Clindamycin	v	v	+	+	+++
Streptomycin	+	v	-	-	++[3]
Cefotaxim	+	+	+	-	+++

[1] +, sensitive; -, insensitive; v, variation in sensitivity between bacterial species (source: McEvoy, GK (Ed): AHFS Drug Information 90, American Society of Hospital Pharmacists, 1990).

[2] +, elimination of SFBs by dose ≥ therapeutic dose (TD); ++, elimination by dose ≥ TD x 10^{-1}; +++, elimination by dose ≥ TD x 10^{-2}.

[3] Sensitivity of SFBs to streptomycin given at a dose TD x 10^{-2} not tested.

A wide range of antimicrobial drugs eliminate SFBs from the murine ileum after their addition to the drinking water (6, 64-67). Table 2 summarizes the observed *in vivo* sensitivity of SFBs to antimicrobial drugs (64), compared to the *in vitro* sensitivity of facultatively anaerobic and obligately anaerobic bacteria. SFBs are inhibited by doses of ciprofloxacin as low as 1% of the therapeutic dose, whereas obligately anaerobic bacteria are insensitive. The latter bacteria are also insensitive to gentamicin and streptomycin, whereas these drugs, given at 10% of the therapeutic mouse dose, inhibited SFBs. Thus, SFBs behave unlike obligate anaerobes. Virginiamycin added to the food significantly reduces SFB appearance in the ileum of chickens (50).

It is clear that *in vivo* studies on SFB colonization require strict control of host characteristics and environmental conditions. SFB colonization is influenced by a wide range of factors, the basis for this being unknown.

III.6. Determinants of SFB survival *in vitro*

So far, SFBs have not been cultured *in vitro*. Perhaps SFBs can be cultured under appropriate conditions. In any event, for the time being, the identification of factors that affect SFBs *in vitro* requires studies on SFB viability as indicated by subsequent colonization of germ-free animals. SFBs do survive during *in vitro* incubation. After 7 days of anaerobic incubation of caecal homogenates from specific pathogen-free mice on agar plates, SFBs were still viable (68, 69). In germ-free mice inoculated with this culture, SFB colonization could be demonstrated (68, 69). Dilutions of homogenates of mouse and rat caeca that were directly inoculated into germ-free mice and rats also produced SFB colonization (32). In another experiment, caecal homogenates were diluted (10x, 100x and 1000x) and inoculated into germ-free mice either directly or after 1 week of anaerobic incubation (69). Inoculation with both the incubated and non-incubated homogenates caused dose-dependent SFB colonization in the recipients (70), indicating that final SFB density was not reached within one week. Similar results were found after inoculation with ileal homogenates (71). This nicely illustrates that the SFBs did not multiply during incubation on the agar plates, but merely survived incubation.

Koopman *et al.* (29, 30) described resistant, transmittable forms of SFBs. These

forms share various characteristics with *Bacillus* and *Clostridium*, such as resistance to dehydration (30), aerobic incubation (57) and exposure to ethanol 70-80% (12, 29, 53, 71) and chloroform 3% (29, 71). Unlike Bacillaceae, SFBs are sensitive to temperatures above 55°C (29).

Margulis *et al.* (35) demonstrated that aerobic as well as anaerobic incubation of heated termite hindgut homogenates on soft agar plates resulted in overnight growth of "*Arthromitus*-like" (SFB-like) bacteria. The filamentous morphology of these bacteria was demonstrated with electron microscopy. Filaments in aerobic incubations tended to be shorter than those in either anaerobic cultures or in the termite hindgut. It would be interesting to see whether murine SFBs behave similarly to termite SFBs under the conditions described by Margulis *et al.* (35).

IV. POSSIBLE BENEFICIAL EFFECTS OF SFBs

SFBs have various characteristics suggesting that they are beneficial to the host. SFBs, or at least morphologically and ecologically similar, apathogenic and autochthonous bacteria, occur in a wide range of hosts. SFBs are attached to the intestinal epithelium with predilection for epithelium covering gut-associated lymphoid tissue (GALT). They are absent in suckling animals, but appear immediately after weaning. SFBs are sensitive to factors that impair g.i. colonization resistance (CR). Below, we hypothesize that these characteristics of SFBs play a role in maintaining host resistance to enteropathogens.

SFBs might competitively exclude pathogens, especially on the epithelium of Peyer's patches where SFB populations are very dense. Such a protective effect of SFBs was first suggested by Fuller and Turvey (72). Later, other researchers also hypothesized that SFBs might block colonization of this epithelium by pathogenic or potentially pathogenic bacteria (3, 15, 62, 73). Such pathogens may be *Salmonella enteritidis* (15, 74), *Salmonella typhimurium* (73, 75, 76) and *Escherichia coli* (75). Indeed, Garland *et al.* (15) reported a positive correlation between *in situ* counts of ileal SFBs in rats and host resistance to *S. enteritidis*. Merrell *et al.* (3) found that hyperbaric stress-induced disappearance of SFBs from the mouse coincided with increases of facultatively anaerobic bacteria in the intestine and of translocation of bacteria to the liver. However, any causality of

these relationships has not been demonstrated.

Hohmann *et al.* (77) suggested that the Peyer's patches, rather than villi, are the primary sites of salmonella uptake. Mayrhofer (78) reported that Peyer's patches are the sites of initial adherence of some pathogenic *E. coli* and also the areas of primary colonization and multiplication by pathogenic salmonellae. Carter and Collins (74), however, found that *S. enteritidis* administered intragastrically rapidly penetrates the villus epithelium and reaches the local Peyer's patches via the lymphatic capillaries. In any case, the ileal Peyer's patches, either as site of primary invasion or as collection point, are probably intimately involved in the host's response to infection by salmonellae.

The preferential adherence of SFBs to the Peyer's patch epithelium might stimulate the GALT. There are no data indicating that SFBs influence either specific or non-specific immunity. It is only known that some components of the autochthonous g.i. microbiota can stimulate non-specific immunity, either systemically (59, 79-82) or locally (18, 81-88) or both systemically and locally (82, 89, 90). Autochthonous gut bacteria can also stimulate specific immunity (91-95) and induce specific immunological tolerance (60, 92, 94, 96-98).

The formation of suppressor T cells takes place in the GALT and these T cells then migrate to peripheral lymphoid tissue such as the spleen to provide systemic tolerance (98). In mice, this specific immunological tolerance is formed between the second and the fourth week of life (97). During this period of postnatal life, SFBs begin to colonize the ileum. No relationship between SFBs and development of immunological tolerance has been demonstrated, but an immunostimulating role for SFBs has been suggested in mice, rats and chickens (8).

It is anticipated that the contribution of SFBs to host resistance by competition with pathogens depends on the density of the SFB populations. Thus the low SFB densities in chickens older than twelve weeks (8, 20) may only influence CR indirectly, i.e. by enhancing mucosal immunity. As to this possibility, many points remain to be settled. What is the nature of this influence? Are either humoral or cellular immune mechanisms involved? How can SFBs affect the GALT and induce immunity to pathogenic bacteria without being attacked by the same immune system? Mice mono-associated with SFBs, which are now available (53), may prove to be of great importance to answer these questions.

External factors that negatively affect SFBs, such as environmental stress and

antimicrobial drugs, also strongly impair g.i. CR. This was clearly shown for host resistance to salmonella (74, 99, 100). These adverse influences in animals with a conventional microbiota are very complex, and to obtain clues as to a causative influence of SFBs on g.i. CR, gnotobiotic mice (mice with a defined microbiota) with and without SFBs should be compared.

It is likely that the SFB-like microbes seen in a wide variety of host species are all ubiquitous symbionts. This could imply they have the same function in different host species. Possibly, this function is providing g.i. CR.

V. CONCLUSIONS

Based on our review of the literature on SFBs we conclude the following.

1. SFBs are highly evolved and host-adapted, autochthonous, apathogenic bacteria being part of the indigenous microbiota in the ileum of mice and rats.
2. SFBs are intimately mucosa-associated symbionts located on the villi and particularly on the Peyer's patches of the ileum.
3. SFBs in the ileum differ in shape and ultrastructure, indicating a complex life cycle.
4. SFB-like bacteria occur in the ileum of myriapods, termites, cockroaches, beetles, isopods, frogs, toads, domestic ducks, domestic fowl, guinea pigs, zebras, dogs, cats, sheep and vervet monkeys.
5. SFBs associate with other autochthonous, intestinal bacteria, which may be a form of symbiosis.
6. SFB colonization varies greatly between apparently identical individuals and from one experiment to another.
7. SFB colonization is influenced by a wide range of host characteristics, diet composition, various forms of stress and various antimicrobial drugs.
8. SFBs have not yet been cultured *in vitro*.
9. SFBs kept under *in vitro* conditions can colonize germ-free animals.
10. SFBs might contribute to host resistance against pathogens, by competition and/or by stimulation of the host's immune system.
11. Mice mono-associated with SFBs, which are now available, may be of great importance to study effects of SFBs on the host.

Fig. 10 presents our concept of possible relationships between SFBs, host and environment. Host and environmental factors do influence SFB colonization, but by unknown mechanisms. It is not known whether SFBs influence host resistance to pathogens. If they do, this might be mediated through stimulation of host immunity and/or induction of immunological tolerance and/or enhancement of g.i. CR.

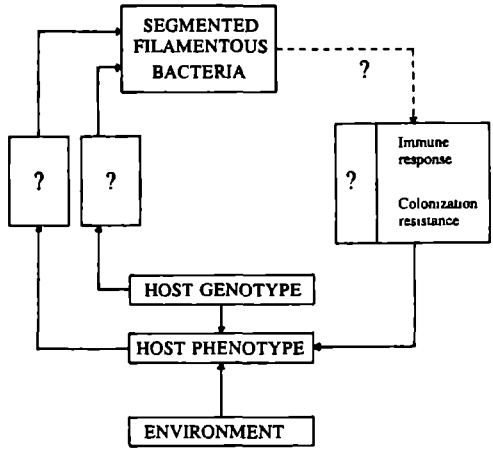

Fig. 10. Schematic presentation of the possible relationships between segmented, filamentous bacteria, host and environment.

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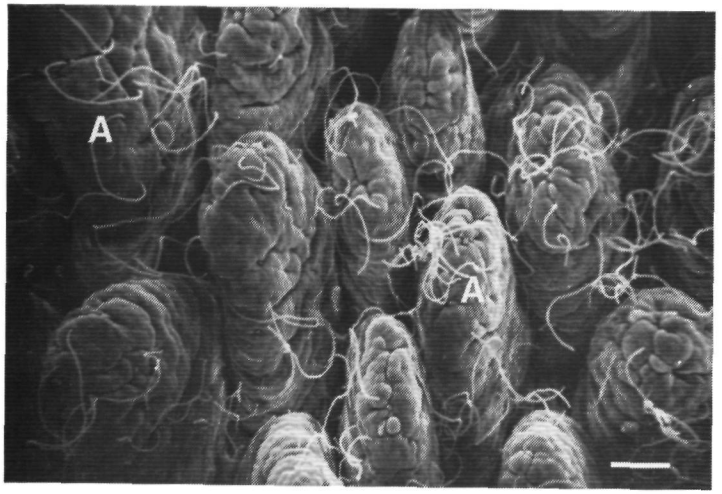

Fig. 1. Scanning electron microscopy of ileal villi of a 25-day-old mouse. (A) Segmented, filamentous bacteria attached to the epithelium. Bar = 31 μm.

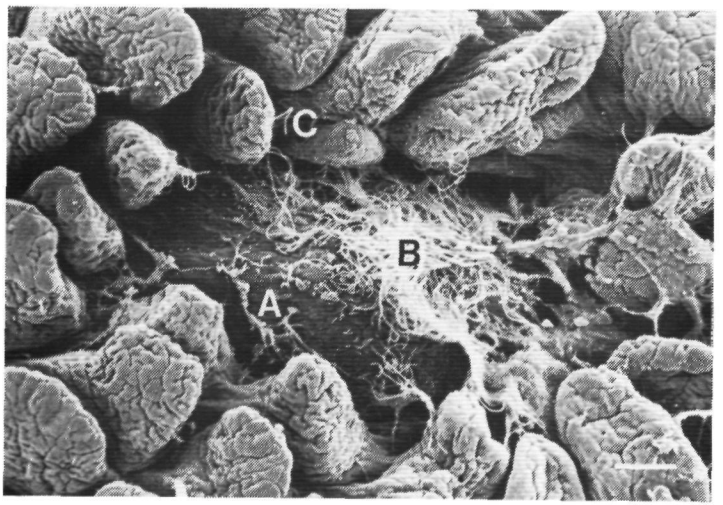

Fig. 2. Scanning electron microscopy of a Peyer's patch and surrounding villi in the ileum of a 25-day-old mouse. (A) Peyer's patch. (B) Peyer's patch-associated, dense population of segmented, filamentous bacteria (SFBs). (C) Single SFB attached to the epithelium of a villus. Bar = 63 μm.

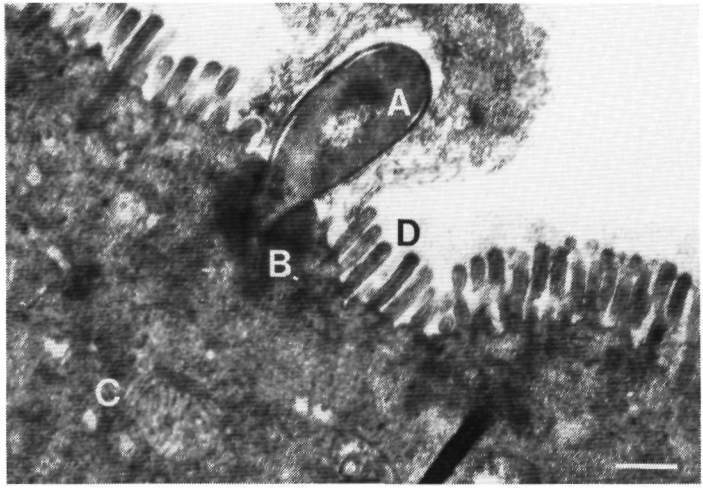

Fig. 3. Transmission electron microscopy of the attachment site of a segmented, filamentous bacterium (SFB) in the ileum of a 25-day-old mouse. (A) Holdfast segment of an SFB. (B) Nipple-like appendage of the holdfast segment. (C) Apical cytoplasm of an epithelial cell. (D) Microvilli. Bar = 0.3 μm.

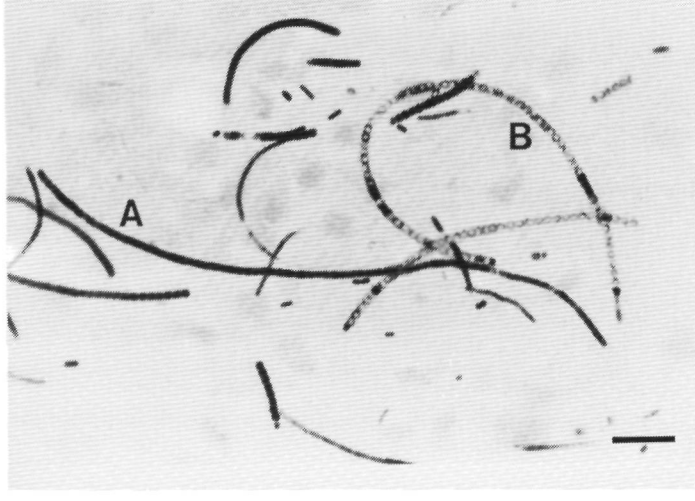

Fig. 5. Light microscopy of a Gram-stained mucosal smear from the ileum of a 25-day-old mouse. (A) Segmented, filamentous bacterium (SFB) without morphological characteristics of differentiation. (B) SFB showing internal differentiation (note the spore-like bodies along the entire length of the SFB). Bar = 12 μm.

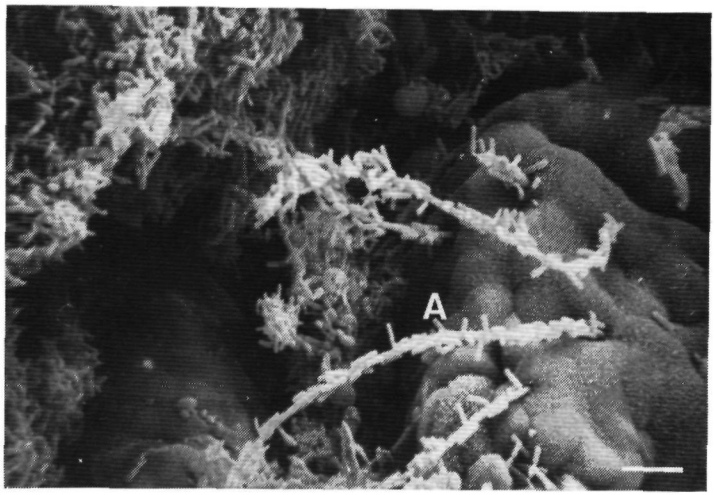

Fig. 6. Scanning electron microscopy of the ileum of an adult mouse. (A) Segmented, filamentous bacterium, almost completely covered with rod-shaped epibionts. Bar = 8 μm.

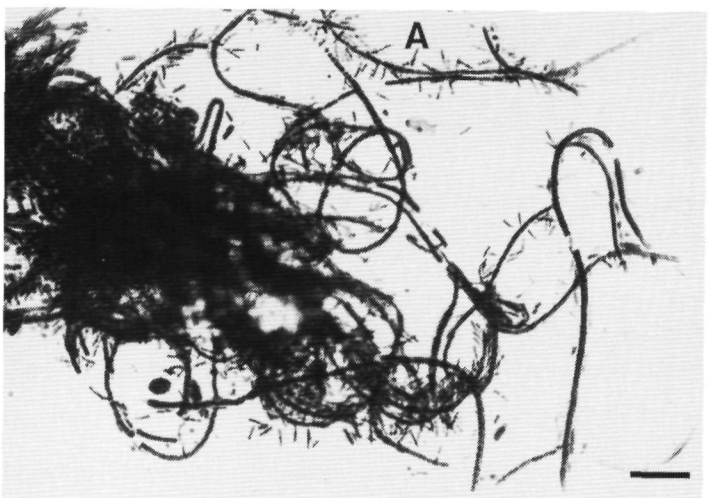

Fig. 7. Light microscopy of a Gram-stained mucosal smear from the ileum of an adult mouse. (A) Segmented, filamentous bacterium subcolonized by rod-shaped epibionts. Bar = 12 μm.

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1.3 Intestinal, segmented, filamentous bacteria occur in a wide range of vertebrate animal species

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SUMMARY

Segmented, filamentous bacteria (SFBs) are known to occur in the intestine of laboratory animals such as mice, rats, chickens, dogs and cats. To see whether this extends to other animal species, the intestines of individuals from 18 vertebrate species, including man, were examined for the presence of SFBs. SFBs were detected with light microscopy in the cat, dog, rhesus monkey, crab-eating macaque, domestic fowl, South African claw-footed toad, carp, man, laboratory mouse and rat, wood mouse, jackdaw and magpie. These results suggest that apathogenic SFBs are ubiquitous in the animal kingdom. Among apparently identical animals, there was considerable variation in the degree of SFB colonization. It is suggested that SFB colonization could serve as an indicator of standardization of animal experimentation.

INTRODUCTION

Segmented, filamentous bacteria (SFBs) are apathogenic, autochthonous microbes in the ileum of mice and rats from various laboratories throughout the world (Davis & Savage, 1974; Chase & Erlandsen, 1976; Garland *et al.*, 1982; Tannock *et al.*, 1984; Koopman *et al.*, 1987). SFBs have never been cultured *in vitro*, and thus never classified and characterized physiologically. They are identified with the use of electron or light microscopy on the basis of their large dimension (up to a length of more than 1 mm), unique morphology (filaments, segmentation, spore-formation) and specific habitat (attachment to the ileal mucosa). A morphological and ecological similarity exists between mouse and rat SFBs, but they represent at least two different bacterial species. Mouse-derived SFBs do not colonize the rat ileum and vice versa (Tannock *et al.*, 1984; Koopman *et al.*, 1984). Mainly on the basis of chance observations, it would seem that SFB-like bacteria occur in the intestine of vertebrates such as frog and toad (tadpole), domestic duck, domestic fowl, guinea pig, zebra, dog, cat, sheep and vervet monkey (*Cercopithecus aethiops*) and in the intestine of invertebrates such as myriapod, termite, cockroach, beetle and isopod (Klaasen *et al.*, in press). Thus, although no systematic investigations were carried out, it appears that SFB-like bacteria are

quite ubiquitous. In the gut of termites, Margulis *et al.* (1990) have observed a group of filamentous, spore-forming bacterial species belonging to the genus *Arthromitus*. *Arthromitus*-like bacteria are probably taxonomically related to the SFB-like bacteria in various vertebrates and invertebrates.

From the point of view of laboratory animal science, SFBs are of considerable interest. They have been suggested to contribute to the colonization resistance of the small intestine to pathogenic bacteria (Merrell *et al.*, 1979; Roach & Tannock, 1979; Garland *et al.*, 1982). The appearance of SFBs in the ileum of mice can vary greatly between apparently identical individuals and group means of SFB colonization for apparently identical groups can vary considerably from one experiment to another (Klaasen *et al.*, 1990 and 1991a). The variability of SFB colonization may well extend to other parameters. Theoretically, standardization of the animals and their environment should reduce the variability of results, but standardization can only involve the sources of variation that we know (Beynen, 1991). Clearly, as to SFB colonization these sources of variation remain partly unknown. Identified sources of variation in mice are diet, strain and housing (Klaasen *et al.*, 1990 and 1991b; Koopman *et al.*, 1989).

The objective of the present study was to systematically examine, with the same technique, the occurrence of SFBs in a wide range of vertebrate species that are used as laboratory animals. The information thus obtained may provide more insight into the significance of this group of bacteria and also into possible factors controlling their colonization. In athymic mice, the population density of SFBs may be lower than in euthymic mice (Davis & Balish, 1979). We therefore investigated selected asymptomatic mice and rats with an impaired immune system. We also microscopically studied for the presence of SFBs human intestinal samples and those from one wild mammal and two wild birds, which were offered for postmortal examination.

MATERIALS AND METHODS

Origin of samples

Intestinal samples from individuals of 14 laboratory animal species were examined.

Tables 1 and 2 present characteristics of the animals studied. The mice and rats were selected on the basis of their specific characteristics. There were two strainsof athymic (nude) mice and one strain of "SCID" mice with a reduced number of lymphokine activated killer (LAK) cells (Kamel-Reid & Dick, 1988). We used a strain of rats (Lew:Han; Lewis) with an increased susceptibility to autoimmune diseases (Holda & Swanborg, 1980) and a Fischer strain (CDF(F-344)CrlBr), which is frequently used as control for the Lewis rats (Davis *et al.*, 1985). The F-344 rnu/rnu strain is a strain of Fischer nude rats. There were two types of Cpb:WU (Wistar) rats with a genetically determined difference in behavioural responses, so-called "freezing" and "fleeing" rats (Cools *et al.*, 1990). To screen the animal species and strains given in Tables 1 and 2, we used control animals from ongoing experiments.

Human intestinal samples were derived from six patients either examined or operated at the University Hospital of Nijmegen. In one patient (no.1), an ileal biopsy was taken. In the other five patients, parts of the gut were surgically removed. None of the patients had been treated with antimicrobial drugs shortly before collection of samples. However, two patients (no. 5 and 6) were treated with corticosteroids prior to resection. Patients' characteristics were as follows (patient no., sex, age in yrs, intestinal disease, parts of the intestine examined for SFBs): 1, male, 67, intestinal polyps, ileum; 2, female, 55, intestinal polyps, ileum plus colon ascendens, transversum and descendens; 3, female, 46, colitis ulcerosa, ileum plus colon ascendens, transversum and descendens; 4, female, 57, colitis ulcerosa, ileum plus colon ascendens and transversum; 5, male, 33, colitis ulcerosa, colon transversum and sigmoid colon; 6, male, 20, Crohn's disease, jejunum plus ileum and caecum.Three wild animals, found in a diseased state or killed by an accident, were examined. There were a wood mouse (*Apodemus sylvaticus*) and a jackdaw (*Corvus monedula*) both discovered in the region of Nijmegen, The Netherlands, and a young magpie (*Pica pica*) from the area near Hamburg, Germany.

Preparation of mucosal smears

The animals given in Tables 1 and 2 were euthanized as follows. Inhalation of carbon dioxide was used for guinea pigs, golden hamsters, mice and rats. The

Table 1. Characteristics of animals examined for the presence of SFBs

Species	Breed or strain	Number of individuals studied (♀,♂)	Age	Origin[1]	Type of housing[2]	Type of feed[3]
Guinea pig (*Cavia porcellus*)	Dunkin Hartley	4, 3	2-8 mo	A	cc	a
Golden hamster (*Mesocricetus auratus*)		15, 12	1½-5 mo	A	cc	b
Rabbit (*Oryctolagus cuniculus*)	New Zealand White	7, 5	1½-9 mo	A	cc	c
Cat (*Felis catus*)	European shorthair	0, 27	6-9 mo	A	c	d
Dog (*Canis familiaris*)	Beagle	1, 4 5, 18	3½-11 y 3-6 mo	A B	c c	e e
Goat (*Capra hircus*)	Saanen	6, 0	1½-2 y	C	c	f
Horse (*Equus caballus*)	Shetland pony	1, 1	20 y	A	c	g
Rhesus monkey (*Macaca mulatta*)		5, 0	2 wk-15½ y	A	cc	h
Crab-eating macaque (*Macaca fascicularis/irus*)		3, 2 10, 13	2 y 15 mo-15 y	A D	cc cc	h h
Domestic fowl (*Gallus domesticus*)	White Leghorn	30, 0 24, 0	8-9 wk 10-15 wk	E F	SPF cc	i i
South-African claw-footed toad (*Xenopus laevis*)		1, 3	5-7 y	A	c	j
Carp (*Cyprinus carpio*)		4[4]	1 y	G	c	j

[1] Explanation of symbols: A, Central Animal Laboratory, Catholic University of Nijmegen, The Netherlands (the cats had been bred under SPF conditions and were derived from ancestors obtained in 1975 from OLAC Western Ltd , Llandeilo, Pyfed, Wales, Great Britain); B, housed at the Central Animal Laboratory, Nijmegen, but obtained at the age of 2½ months from Intervet International BV, Boxmeer, The Netherlands; C, various goat breeding farms in The Netherlands; D, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands; E, Intervet International BV, Boxmeer, The Netherlands; F, Poultry Health Institute, Doorn, The Netherlands (chickens derived from various commercial breeders in The Netherlands); G, Biological Institute, Catholic University of Nijmegen, The Netherlands.

[2] Explanation of symbols: cc, clean-conventional; c, conventional; SPF, specified pathogen-free.

[3] Explanation of symbols: a, guinea pig diet LC23-B; b, rat-mouse-hamster diet RMH-TM; c, rabbit diet LK-04; d, cat diet LF-32; e, dog food D.B.; f, goat pellet; g, grass and hay; h, primate diet G.O.; i, various commercial diets; j, trout food. Diets a-e and h were obtained from Hope Farms BV, Woerden, The Netherlands, diet f from Sluis, Veghel, The Netherlands, and diet j from Trouw Diervoeders BV, Putten, The Netherlands.

[4] Sex unknown.

Table 2. Characteristics of mice and rats screened for the presence of SFBs

Species	Strain	Supplier[1]	Age	Immunological state	Type of housing[2]	Type of feed[3]
mouse (*Mus musculus*)	BALB/c ABom nu/nu	A	1-7 mo	athymic	bm	a
mouse	Crl:CD-1 (ICR)BR nu/nu	B	2½-5½ mo	athymic	bm	a
mouse	Crl:NIH3BR ("SCID")	B	5 mo	few LAK cells	bm	a
rat (*Rattus norvegicus*)	LEW:Han	C	4½-5 mo	euthymic	cc	b
rat	CDF(F-344) CRlBR	B	5 mo	euthymic	cc	b
rat	F344 rnu/rnu		5 mo	athymic	bm	a
rat	Cpb:WU	E				
	"freezing rats"		3-9 mo	euthymic	cc	b
	"fleeing rats"		idem	idem	idem	idem

[1] Explanation of symbols: A, Bomholtgaard Breeding and Research Center Ltd., Ry, Denmark; B, Charles River Wiga GmbH, Sulzfeld, Germany; C, Zentralinstitut für Versuchstierzucht, Hannover, Germany; D, Harlan OLAC Ltd., Bicester, Great Britain; E, Home-bred Cpb:WU rats derived from ancestors obtained in 1980 from the central laboratory animal facility of TNO, Zeist, The Netherlands. The production and characteristics of the "freezing" and "fleeing" rats have been described by Cools *et al.* (1990).

[2] Explanation of symbols: bm, barrier-maintained; cc, clean-conventional.

[3] Explanation of symbols: a, SRM-GS; b, RMH-TM. The diets were purchased from Hope Farms BV, Woerden, The Netherlands.

rabbits, cats, dogs, goats, ponies, rhesus monkeys and crab-eating macaques were killed by intravenous administration of an overdosis of barbiturates. Domestic fowl were killed by cervical dislocation, and toads and carps by decerebration. The distal half of the small bowel and the complete caecum (if present) were removed. From three dogs and two goats, palatine tonsils were also removed, and from the South African claw-footed toads, carps, jackdaw and magpie, the colon and rectum were collected.

Each tonsil was removed from the oral cavity and with its mucosal side vigorously rubbed on a microscopic slide over a surface of 3 cm^2. Then, each tonsil was cut into three equal parts; all sectioning surfaces were similarly rubbed on a slide. The intestinal parts to be examined were opened lengthwise. The contents were gently removed with a pair of tweezers. Pieces of gut wall, each with a surface of circa 1 cm^2, were removed. There was 1 piece per 1-10 cm of gut, depending on the length of the gut. The method used for the preparation of small intestinal samples from mice and rats has been described earlier (Koopman *et al.*, 1986). Each piece of gut wall was rubbed with its mucosal side on a slide as described above. Tonsillar and intestinal mucosal smears were fixed by dry heat and Gram-stained.

Light microscopy

Bacteria with SFB-like morphology were identified by light microscopic examination of the mucosal smears at a magnification of 1000x. In each smear, 100 fields divided over 5 randomly chosen spots of the slide (20 fields per spot) were examined. The incidence of SFB-positive animals was determined. For individual mice and rats, the mean number of SFB-positive fields per smear (SFB score, ranging from 0-100) was calculated. For mice with SFB scores ranging from 20 to 60, the within-smear variation of positive fields/20 fields is on average 30 % (coefficient of variation). Light micrographs of SFBs in mucosal smears were made with a Leitz Orthoplan^R photo microscope.

Scanning electron microscopy

From selected SFB-positive animals, samples of gut wall were taken to examine the

mucosa and adhering SFBs with the use of scanning electron microscopy. These samples were obtained as described above, flushed with a 0.9 % (w/v) NaCl solution, stretched on filter paper and fixed for 24h at 20°C in a solution of 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4, 320 mOsmol). After a brief rinse with 0.1 M cacodylate buffer (pH 7.4, 20°C), the specimens were post-fixed for 3-5h at 20°C in Palade fixative (2% OsO_4 in 0.6 M veronal acetate buffer, pH 7.4). Thereafter, they were dehydrated in a graded series of ethanols or acetones and critical-point dried. After coating with a 30 nm gold layer in a Polaron E5100^R sputter coater, the specimens were studied in a Philips SEM 500^R scanning electron microscope. Micrographs were taken at 12-15 kV and at magnifications varying between 160x and 3000x.

RESULTS

For 12 vertebrate species, the incidence of SFB-harbouring animals as based on light microscopic findings, is given in Table 3. SFB-positive animals were found in the following species tested: cat, dog, rhesus monkey, crab-eating macaque, domestic fowl, South African claw-footed toad and carp. Figures 1-7 show light or scanning electron micrographs of selected, SFB-positive individual animals. In all SFB-positive animals, except for 19 out of the 52 chickens, SFBs were detected in the small bowel. All SFB-positive chickens had SFBs in the caecum (Table 3). In two of 9 positive dogs, SFBs were not only detected in the small bowel, but also in a smear from the tonsillar mucosa. The positive toad showed SFBs both in the small bowel and colon (Table 3). The location of SFBs in carp was either the small bowel or colon.

In the SFB-positive dogs and crab-eating macaques, SFBs were found in both sexes. The group of four toads with one positive male, had only one female (Table 3). The numbers of animals examined were too small to demonstrate sex differences in SFB colonization, if any. The SFB scores of positive animals in Table 3 varied between 1 and 79. Within groups of positive animals, there was no uniform SFB localization pattern in the small intestine (data not shown).

Light microscopic examination of the distal small intestine of mice and rats from various strains showed a higher incidence of SFBs in mice than in rats (Table 4).

Table 3. Incidence of SFB-positive animals from 12 vertebrate species

Species[1]	Origin[2]	SFB-positive animals				
		Number/total number of animals				%
		Small bowel ♀	Small bowel ♂	Caecum	Other sites	
Guinea pig	A	0/4	0/3	0/7		0
Golden hamster	A	0/15	0/12	0/27		0
Rabbit	A	0/7	0/5	0/12		0
Cat	A		1/27	0/27		4
Dog	A	1/1	1/4	0/4	2/3[3]	40
	B	3/5	4/18	0/23		30
Goat	C	0/6		0/6	0/2[3]	0
Pony	A	0/1	0/1	0/2		0
Rhesus monkey	A	1/5		1/5		20
Crab-eating macaque	A	0/3	0/2	0/5		0
	D	3/10	3/13	2/23		26
Domestic fowl	E	22/30		30/30		100
	F	13/24		22/24		91
S-African claw-footed toad	A	0/1	1/3	n.c.[4]	1/4[5]	25
Carp	G	4/10[6]		n.c.	4/10[5]	40

[1] Characteristics of animals studied are described in Table 1
[2] See Table 1
[3] Tonsillar mucosa
[4] n.c. = no caecum
[5] Colonic mucosa
[6] Sex unknown

Table 4. SFB colonization of the small bowel in animals from various mouse and rat strains

Species[1]	Strain[1]	SFB-positive animals		SFB score[2]	
		Number/total number of animals	%	X	Range
Mouse	BALB/c ABom nu/nu	19/19	100%	26	2-67
Mouse	Crl:CD-1 (ICR)BR nu/nu	5/5	100%	26	8-60
Mouse	Crl:NIH3BR xid/xid ("SCID")	4/4	100%	32	6-39
Rat	LEW:Han	3/8	38%	1	0-3
Rat	CDF(F-344)CrlBR	3/5	60%	2	0-4
Rat	F344 rnu/rnu	3/5	60%	4	0-9
Rat	Cpb:WU				
	"freezing" rats	10/14	71%	6	0-20
	"fleeing" rats	10/14	71%	4	0-22

[1] Characteristics are given in Table 2
[2] Number of SFB-positive fields per 100 fields as examined in each of five mucosal smears (magnification, 1000x)

For each mouse strain tested, the incidence was 100%. For the rat strains tested,the incidences ranged between 38 and 71%. Mean SFB scores were higher for the mice than rats.

One of the six human patients tested, had SFBs in the ileum as shown by light microscopy (Fig. 8). From the positive patient, an ileal biopsy without abnormalities had been obtained. The three wild animals examined (wood mouse, jackdaw and magpie) had SFBs in the small intestine, as demonstrated by light microscopy (Fig. 9-11).

Examination of intestinal samples from animals of 13 out of the 18 species tested, revealed the occurrence of SFBs. There was a great variation of the degree of SFB colonization between and within species. SFBs have been shown to attach preferentially to ileal Peyer's patches or caecal tonsils, suggesting a functional relationship between SFBs and mucosa-associated lymphoid tissue (Abrams, 1977; Glick *et al.*, 1978; Owen & Nemanic, 1978; Garland *et al*, 1982; Tannock *et al.*, 1984). However, in asymptomatic mice and rats with an impaired immune system, relatively large numbers of SFBs were found. Colonization density of SFBs was higher in the mice than in the rats examined. The idea of the possible relationship between SFBs and lymphoid tissue is supported by the observations that in dogs SFBs were associated with tonsillar mucosa, and in chickens with caecal mucosa.

The present study supports the chance observations that SFB-like bacteria can occur in a wide variety of vertebrate host species (Klaasen *et al.*, in press). As far as we know, this study documents for the first time that SFBs may not be found in hamster, rabbit, goat and pony, whereas they may be found in man, rhesus monkey, crab-eating macaque, South-African claw-footed toad, carp, wood mouse, jackdaw and magpie. The light microscopic observation of SFBs in man, rhesus monkey and crab-eating macaque is supported by the scanning electron microscopic observation of SFBs in the vervet monkey (Bruorton, 1991), which is also a primate species. However, our observations have to be interpreted carefully because the microbes found in the latter 8 species have not been examined by scanning or transmission electron microscopy. It should also be emphasized that unability to detect mucosa-associated SFBs could relate to the gut being only poorly or irregularly colonized by SFBs (Davis & Balish, 1979). When sampling of SFB-positive animals takes place more than 3 h postmortem SFBs may not be detected (Davis, 1980; own unpublished observations). Except for the SFB-positive sample, the other human ileal samples were derived from diseased intestines. Thus, the low incidence of SFBs in the group of six patients may not be representative.

Colonization of SFBs is dependent on a great number of host-related and environmental factors (Klaasen *et al.*, 1990, 1991b and d; idem, in press). We therefore described the characteristics of the animals examined as carefully as possible (Tables 1 and 2). However, even in apparently identical individual animals

or groups of animals, SFB colonization can differ considerably (Klaasen *et al.*, 1990 and 1991a). Possibly, the variation of SFB colonization in apparently identical animals may serve as an indicator of standardisation of animal experimentation. Up until now, the sources of variation of SFB colonization are not well-known. It could be suggested that reproducibility from one experiment to another of an extremely variable parameter such as SFB colonization (SFB score) normally is, entails reproducibility of other parameters as well.

Thus, mucosa-associated, SFB-like bacteria occur in various host species, including man and at least two other primate species. Margulis *et al.* (1990) have identified 22 microscopically distinguishable, symbiotic arthromitids in 9 different arthropod hosts. Possibly, in the course of evolution, symbiotic intestinal SFBs evolved together with their hosts into a stable mutualistic relationship. Biochemical studies and bacterial DNA/RNA analysis will be necessary to unravel the taxonomy of SFBs. SFBs do not disappear, nor do they cause clinical symptoms when their host lacks certain immunological functions. We may therefore conclude that SFBs are apathogenic, ubiquitous, autochthonous gut inhabitants. This raises the question of their significance to the host. With the use of germ-free and SFB-mono-associated mice (Klaasen *et al.*, 1991c), the influence of SFBs on gastro-intestinal colonization resistance and mucosal immunity can be investigated. If SFBs appear to be beneficial to the host, then investigations on human SFBs may become relevant.

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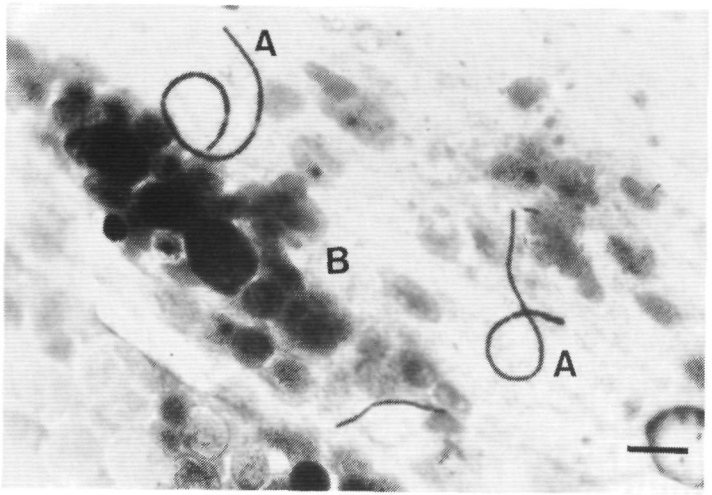

Fig. 1. Light microscopy of a mucosal smear from the ileum of a cat. (A), Segmented, filamentous bacterium (SFB). (B), Gut material consisting of epithelial cells and mucus. Bar = 12 μm.

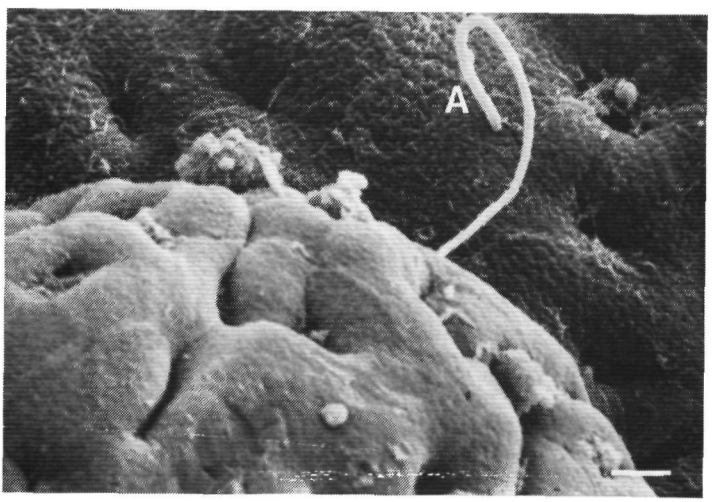

Fig. 2. Scanning electron microscopy of the tip of an ileal villus of a cat. (A), SFB attached to the villous epithelium. Bar = 16 μm.

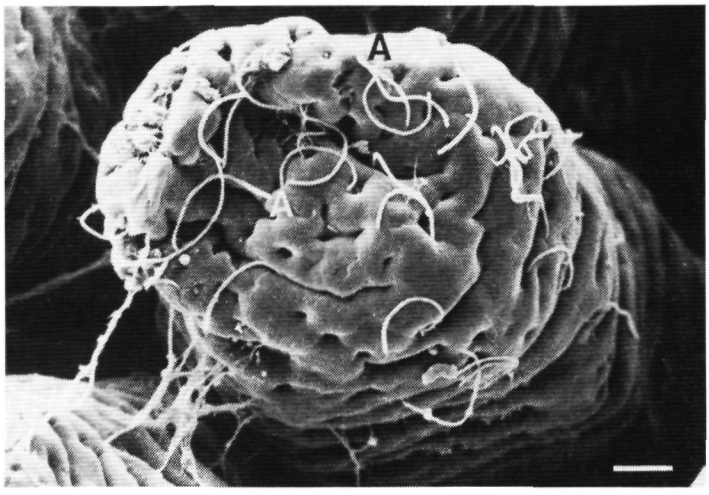

Fig. 3. Scanning electron microscopy of an ileal villus of a dog. (A), Attachment sites of SFBs. Bar = 16 μm.

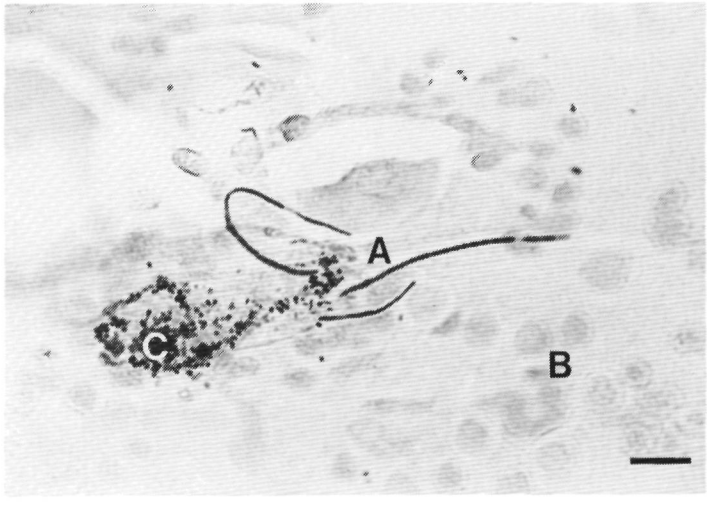

Fig. 4. Light microscopy of a smear from the tonsillar mucosa of a dog. (A), SFB. (B), Tonsillar material consisting of epithelial cells and mucus. (C), Accumulation of cell debris and bacteria. Bar = 12 μm.

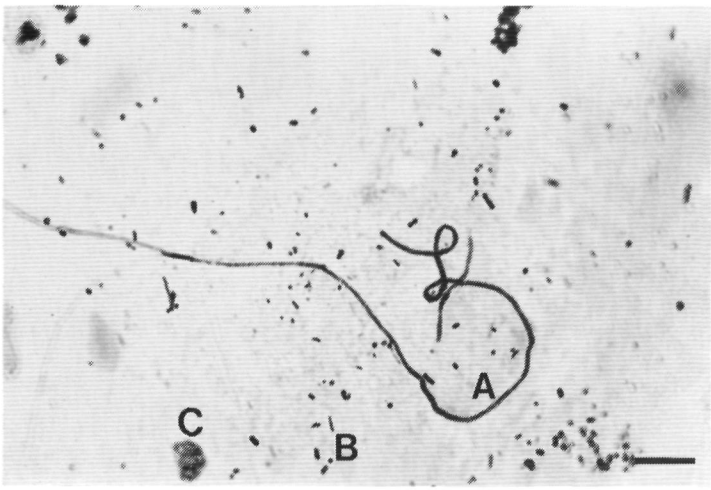

Fig. 5. Light microscopy of a mucosal smear from the ileum of a crab-eating monkey. (A), SFB. (B), Rod-shaped and coccoid, intestinal bacteria. (C), Mucosal material. Bar = 12 μm.

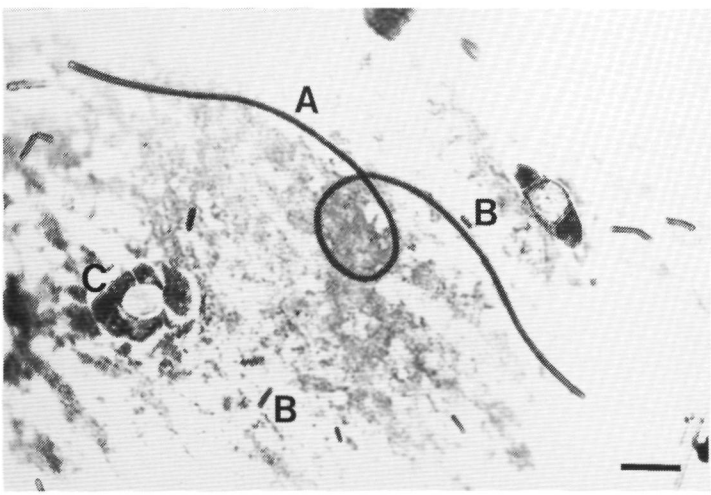

Fig. 6. Light microscopy of a mucosal smear from the small intestine of a South-African clawfooted toad. (A), SFB. (B), Rod-shaped, intestinal bacteria. (C), Damaged epithelial cell. Bar = 12 μm.

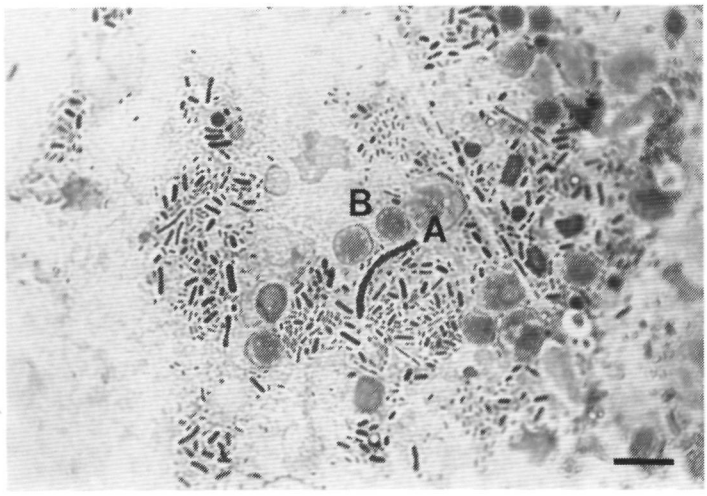

Fig. 7. Light microscopy of a mucosal smear from the colon of a carp. (A), SFB. (B), Mucosal material consisting of epithelial cells and mucus. Bar = 12 μm.

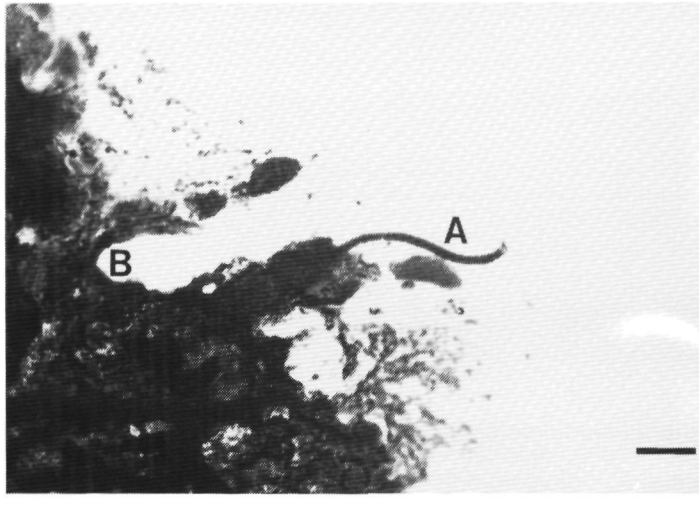

Fig. 8. Light microscopy of a mucosal smear from the ileum of a human adult. (A), SFB attached to the epithelium. (B), Mucosal material consisting of epithelial cells and mucus. Bar = 12 μm.

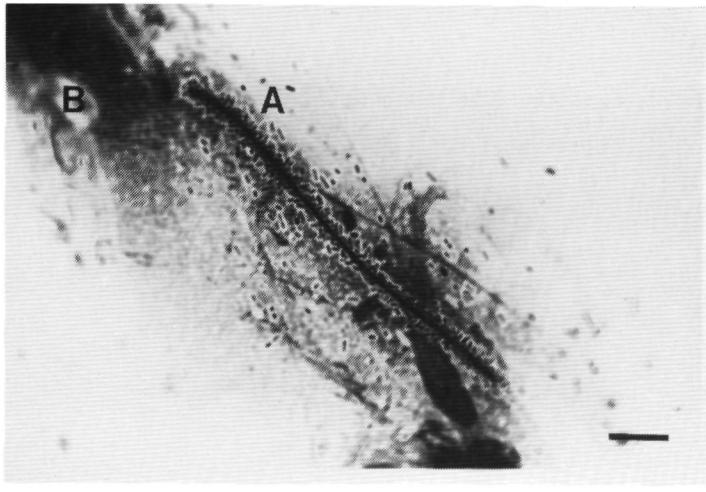

Fig. 9. Light microscopy of a mucosal smear from the ileum of a wood mouse. (A), SFB surrounded by numerous rod-shaped bacteria. (B), Mucosal material consisting of epithelial cells and mucus. Bar = 12 μm.

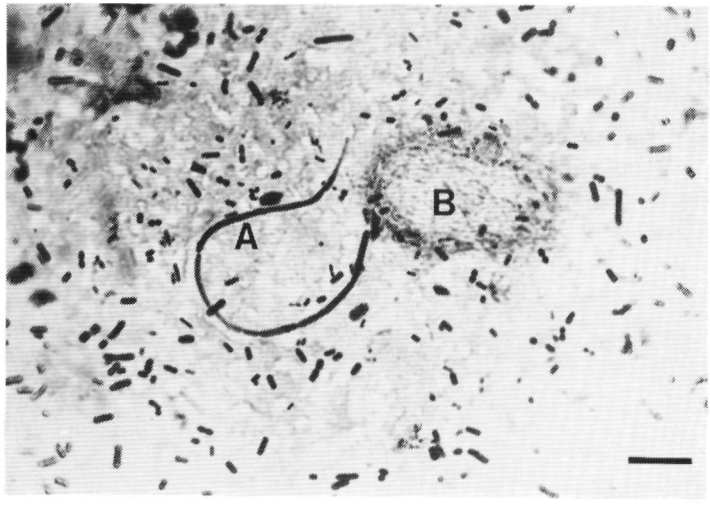

Fig. 10. Light microscopy of a mucosal smear from the small intestine of a jackdaw. (A), SFB. (B), Mucosal material consisting of epithelial cells and mucus. Bar = 12 μm.

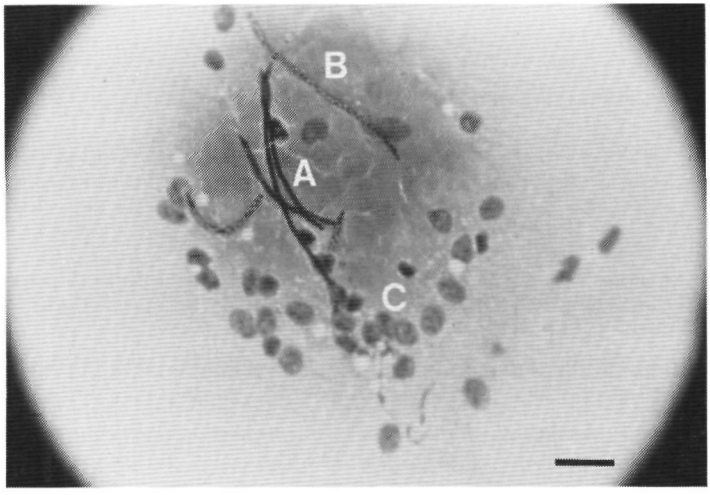

Fig. 11. Light microscopy of a mucosal smear from the small intestine of a magpie. (A), SFB, slender and dark-coloured. (B), SFB, broader and light-coloured. (C), Mucosal material consisting of epithelial cells and mucus. Bar = 12 μm.

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Chapter 2

Determinants of colonization of murine ileum by SFBs

2.1 Effect of preventing coprophagy on colonization by segmented filamentous bacteria in the small bowel of mice

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SUMMARY

Segmented filamentous bacteria (SFBs) are present in the small intestine of mice and rats. It is not clear whether this presence is due to a stable colonization or to reinfection by coprophagy. To answer this question the effect of preventing coprophagy on SFBs was studied in mice. Two parameters were determined as general indicators for a normal micro-ecological state of the intestine: the relative caecal weight and the percentage of caecal fusiform shaped bacteria. Prevention of coprophagy executed by means of a polyethylene 'restrainer' and a wire mesh resulted in a slight increase in the colonization of SFBs (significant in small intestine section 8; $P<0.012$). The number of SFBs per 100 bacteria showed an increase in section 8 compared to mice with a restrainer, but which were housed on sawdust ($P<0.032$). No effects were seen on the relative caecal weight and the percentage of fusiform shaped bacteria in the caecum.

INTRODUCTION

From an ecological point of view the segmented filamentous bacteria (SFBs) are very interesting microorganisms.[2] They are strongly associated with the ileal wall of rats and mice and possibly contribute to the resistance of the host to invading bacterial pathogens.[2,4,15]

The SFBs consist of many segments (up to 90) which during the life-cycle either remain undifferentiated or differentiate according to specific morphology and function.[1] Reproductive segments can generate elements which attach to the intestinal epithelium and endospore-like elements which presumably play a role in the spread of the microorganism from one host to another via the faeces.[1,2,12,16,17]
The life-cycle of the SFBs is not entirely clear. To maintain the SFB population in the last part of the small intestine, a continuous recolonization by attachment of SFB elements to the epithelial cells at the basis of the villi is necessary. Because of the constant extrusion of these epithelial cells, which have a turn-over time of 2d, either a stable colonization of SFBs or reinfection by coprophagy must occur.

Here we present the effects of preventing coprophagy on the colonization density of SFBs, in absolute and relative numbers. The only way of examining the

presence and numbers of SFBs is to use a (sub)microscopic method, since cultivation of the bacterium *in vitro* has failed to the present day.[12,15,17]

In order to measure possible disturbances in the gastro-intestinal flora, two indicator parameters are used: relative caecal weight and percentage of fusiform shaped bacteria in the caecum; in combination these two parameters are reliable indicators of the quality of the murine intestinal flora.[6,7]

MATERIALS AND METHODS

Animals

Female Cpb:SE (Swiss) 7wk-old mice with an SPF flora[5] were used. Two similar experiments were carried out. In the first experiment the mice were fed RMH-TM^R pellets (rat/mouse/hamster-test and maintenance; Hope Farms BV, Woerden, The Netherlands) and in the second experiment they were fed a home-made autoclaved diet.[14] Food and tap water were supplied *ad libitum*. The animals were housed individually in macrolon cages type III (RUCO Metaalindustrie Nederland BV, Valkenswaard, The Netherlands). The room temperature varied between 20 and 22°C, while the relative humidity varied from 60 to 70 %. The light regime included 12h of light and 12h of darkness. These housing circumstances were in line with the Dutch legislation.

Method to prevent coprophagy

To prevent coprophagy a restrainer was used made from a polyethylene flask (length 54 mm, diameter 27 mm). It consisted of a neck ring (inner diameter about 12 mm) and a dorsal part (length about 25 mm).[3] Ebino's method[3] was modified in two minor ways: i) a third fixation of the restrainer to the mouse's skin was applied, namely in the neck, and ii) nylon was used as fixation material instead of silk.

In each experiment twelve animals with a restrainer were placed individually in cages (see under *Animals*), six on sawdust and six on a wire mesh. The same was done with 12 animals without restrainer. The combination 'no restrainer-sawdust' served as a negative control for prevention of coprophagy; the combinations 'no

restrainer-wire mesh' and 'restrainer-sawdust' were used to determine the possible influence of a restrainer and that of a wire mesh on the intestinal flora.

Parameters measured

On days 0,1,2,3,5,7,9,11,13 and 14 the body weight of each mouse was determined. The animals were checked daily for loose fixations or restrainers, injuries, disease or mortality, behaviour, etc., and on day 14 they were killed by cervical dislocation and weighed.

The colonization density of SFBs in the small intestine was measured by means of Gram-stained smears of the mucosa prepared as described by Koopman *et al.*[9] Of the nine sections of small intestine defined by this method, the last five (sections 5-9) were examined. In each of these, 20 randomly chosen fields with a magnitude of 1000x, were studied for the presence of SFBs; thus 100 fields per mouse were investigated. For each section this procedure was followed and the examination was carried out by one person. The colonization density for each mouse was expressed in the number of positive fields (SFB score). In addition, the percentage of SFBs within the total bacterial population was determined in each of the last two sections (sections 8 and 9) by counting the number of SFBs per 100 bacteria in a mucosal smear. The caeca with contents were removed and weighed after which the relative caecal weights (RCW = caecal weight in grams/body weight in grams x 100 per cent) were determined.

Finally, the number of caecal fusiform shaped bacteria ('fusiforms') in a sample of 50 bacteria in a Gram-stained smear of caecal contents was expressed as percentages.

Statistical analysis

SFB-scores (sections 5-9, section 8 and section 9) were analyzed with analysis of variance (ANOVA) using diet, group (A,B,C,D) and an interaction term as factors. By interaction is meant that the between group differences are influenced by the diet. The same method was used for the percentage of SFBs in section 8 and section 9, for the RCW and for the percentage of fusiforms. So in all cases statistical analysis was done separately for both experiments.

RESULTS

Daily controls of the behaviour and state of health of the animals did not show any serious disturbance or abnormality. Fixation of the restrainers remained intact, implying that coprophagy was prevented. During both experiments the body weights increased in all groups, with 5-28% of the initial body weights. The colonization density of SFBs per group of mice (SFB-score) is expressed for sections 5-9, for section 8 and for section 9 in Table 1. Presented are mean values calculated by pooling the values of both experiments (RMH-TM^R and home-made diet). For all three parameters the mean values are highest in the non-coprophaging group (B), and in section 8 this increase is statistically significant ($P<0.012$). Studying the effects of restrainer and floor type on the percentages of SFBs in section 8 and 9, a significant difference is seen between group B (mean % SFBs for both experiments and both sections is 25.9%) and group D (6.9%; $P<0.032$).

Mean values of the RCW and of the percentage of fusiforms are presented in Table 2. For both the RCW and the percentage of fusiforms there is a statistically significant difference between the two experiments (in both cases $P<0.001$); between the four groups there are no differences in these two parameters.

Table 1 SFB scores in small intestine sections 5–9 section 8 and 9

Experimental conditions				SFB scores*		
Group	Wire mesh	Restrainer	n	Sections 5 9	Section 8	Section 9
A	+	−	12	34±20	48±31	64±32
B	+	+	11	52±14	81±18	89±14
C	−	−	12	41±16	57±23	73±26
D	−	+	9	35±16	46±30	73±25

*Based on the presence of SFB s in 100 fields (sections 5 9) or in 20 fields (sections 8 9) with a magnitude of 1000 × presented are the percentages of positive fields (mean ± standard deviation)

Table 2 Relative caecal weight (RCW)* and % caecal fusiforms*

			Diet RMH TM®			Diet home-made		
Group	Wire mesh	Restrainer	n	RCW	Fusiforms (%)	n	RCW	Fusiforms (%)
A	+	−	6	1 3±0 2	93 3± 2 7	6	2 7±0 4	96 8±3 4
B	+	+	5	1 7±0 3	86 4± 6 5	6	2 2±0 5	97 3±1 6
C	−	−	6	1 4±0 3	89 0± 7 1	6	2 5±0 4	93 0±7 2
D	−	+	4	1 4±0 1	81 5±14 5	5	2 0±0 2	99 2±1 1

*Mean ± standard deviation

Prevention of coprophagy following the method of Ebino *et al.*[3] did not cause a decrease in the colonization density of SFBs in the small intestine. On the contrary, a slight increase occurred (statistically significant in section 8). The relative numbers of SFBs did not change clearly; only between restrained animals on a wire mesh and those on sawdust did a significant difference exist (section 8).

The absence of major stress factors is of special importance in this experiment, because the SFB colonization in the small bowel of stressed mice is likely to decrease.[13] Ebino *et al.*[3] concluded that fixation of restrainers combined with housing on a wire mesh does not significantly influence mouse behaviour (eating, drinking, exploring, sleeping) nor physiological parameters like growth. Our findings confirm this; therefore we may assume that stress due to the fixation was minimal.

Departing from the assumption that besides blocking coprophagy no other important effect was carried out on the intestinal flora, the SFB scores indicate that the SFB population in the murine ileum at least remained intact after 14 days without coprophagy. Since the renewal process of intestinal epithelial cells in mice lasts two days, a recolonization of epithelial cells with SFBs must have occurred within this period of 14 days.[2]

Therefore, we can draw the conclusion that a stable local (re)colonization of SFBs instead of coprophagy plays the most important role in maintaining the SFB population in the dynamic small intestine. By a reduced oral uptake of (intestinal) bacteria from the environment, as a consequence of blocking coprophagy, a situation may be caused in the small bowel in which there is less competition for space and nutrients, so that the SFB population can develop more strongly. However, a supply of bacteria with the food (in both experiments supplied under non-sterile conditions) was present during the 14 day experimental period. Subsequently a second conclusion from this investigation could be that, since there still is a significant increase of SFBs (absolute and relative) in section 8, these microorganisms must have taken advantage locally, in a period of restricted 'invasion' of other bacteria.

As appears from the results, restrained mice on sawdust had low relative numbers of SFBs compared to the three other groups. Because the absolute

numbers of SFBs did not decrease dramatically in these animals, an increase of 'other bacteria' must have taken place. Since housing on sawdust cannot be responsible for this increase (because the *non*-restrained mice on sawdust did not show such an increase of 'other bacteria'), there is no good explanation for this. Maybe interactions between restrainer and floor type play a role, so that combinations of these two factors cause more complex effects.

As appeared in previous experiments with mice, the SFB colonization can be influenced by the mouse diet, possibly by the presence of unknown antimicrobial components.[9,10] Subsequently in our study a commercial diet (RMH-TM^R) was compared to a semisynthetic (home-made) diet which previously seemed to have some stabilizing effects on the SFB population seen in various pilot experiments. No difference was seen between SFB scores after 14 days of feeding with the commercial and the home-made diet. Apparently in the small bowel there were no disturbing effects of antimicrobial dietary factors. However, the home-made diet caused higher RCWs (> 2%), while the RCW of SPF mice generally varies between 1 and 2%.[8,11] The marked differences in the values of the RCW as well as in those of the percentage of caecal fusiforms between the two experiments can possibly be explained by differences in composition of the two diets. This seems to be supported by data concerning the influence of diet components on intestinal microecological parameters, collected after a series of diet experiments with SPF mice (unpublished observations). Considering our findings, it is clear that the SFB population in the murine ileum - in the absence of antimicrobial substances in the food - is maintained by a stable (re)colonization on a certain level which also depends on the rate of competition with other intestinal bacteria.

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2.2 Effects of age, strain and social hierarchy on colonization by autochthonous segmented filamentous bacteria in the ileum of mice

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INTRODUCTION

One source of irreproducibility of results in biomedical research could be variation in the gastrointestinal microbiota of specific pathogen free (SPF) animals.[11] Extremely variable within and between experiments is the segmented filamentous bacterium (SFB) that lives autochthonously in the ileum of mice.[5,6] So far, the significance of SFBs as to microecology and colonization resistance of the gastrointestinal tract is unknown. However, it has been demonstrated that the population density of SFBs at the ileal mucosa of mice can be influenced by characteristics of the host as well as by its environment.[1,6,8-10,12] Maybe SFB colonization can be used as an indicator of the degree of variation in gastrointestinal microbiota.

In this paper, we describe that strain, age and social hierarchy influence the colonization density of SFBs in the ileum of mice. This may in part explain the variation in the SFB colonization density observed frequently.[3,6]

MATERIALS AND METHODS

Animals and housing

To determine the effects of strain and age we used female Cpb:SE (Swiss) and BALB/c mice, both with an SPF flora.[9] The mice were aged 1-12 mths. They were kept in wire-topped macrolon type III cages (RUCO Metaalindustrie Nederland BV, Valkenswaard, The Netherlands), 4-6 animals per cage.

To study the influence of social hierarchy, 7wk-old male Cpb:SE mice (SPF[9]) were used. They were housed either individually or in groups of three animals, in wire-topped macrolon type II cages. As bedding material sawdust was used throughout. Room temperature was 19-23°C, relative humidity 50-70% and light was on from 06.00-18.00 h. All animals had been fed a home-made pelleted diet[7] since weaning at the age of about 3 weeks and were supplied with tap water *ad libitum*.

Determination of social status

The mice housed 3 per cage were observed 4 times a day for 10 min during 4 consecutive days. Social status of each mouse was determined on the basis of the number of observations during which the animal showed aggressive behaviour or was lying separately. This number was expressed as percentage of the total number of observations. A mouse was classified as dominant if this percentage was 40 or higher. Non-aggressive mice housed in the same cage as a dominant one were designated as subordinate. Indifferent mice were those housed in groups without dominant animals.

Parameters

The animals were killed by cervical dislocation and the colonization density of SFBs in the distal half of the small intestine (SFB score) was determined as described by Klaasen *et al.*[2] In addition, the incidence of SFB-positive mice was determined. In the experiment on the influence of social hierarchy, individual body weights were measured at the beginning and the end of the experiment and changes expressed as percentages of initial values. After killing of the animals, caecal weight was recorded and expressed as percentage of body weight. Individual SFB scores were determined. The percentage of caecal fusiform-shaped bacteria (fusiforms) was determined in Gram-stained smears of caecal contents. The concentration of Enterobacteriaceae in individual faecal samples at the beginning and the end of the experiment was determined according to Koopman *et al.*[4] Relative caecal weight, caecal fusiforms and faecal Enterobacteriaceae served as general indicators of the intestinal microbiota.

RESULTS

In Cpb:SE mice aged 1 - 2½ mths, mean SFB scores varied from 23 to 83. The incidence of SFB-positive mice was 100% (Table 1). Cpb:SE mice older than 2½ mths showed lower SFB scores and reduced incidences (Table 1). BALB/c mice, in which SFB scores and incidences were lower than in Cpb:SE mice of the same

age, showed this age dependency too.

In Table 2 the effects of social status on SFB score, change in body weight, relative caecal weight and percentage caecal fusiforms are presented. SFB scores in subordinate mice were significantly lower than in dominant, indifferent or individually housed mice. Subordinate mice showed weight loss, whereas the other animals gained weight. Relative caecal weight and percentage caecal fusiforms (Table 2) and concentration of faecal Enterobacteriaceae (data not shown) were not influenced by social status.

Table 1: Influence of Strain and Age on Colonization of SFB s in the Ileum of Female Mice

	strain			
	Cpb SE (Swiss)		BALB/c	
age (months)	SFB score[1]	incidence of SFB-positive mice[2]	SFB score[1]	incidence of SFB-positive mice[2]
1	66 ± 11	6/6	n d[3]	n d.
1 5	83 ± 12	12/12	9 ± 12	2/5
2	23 ± 15	24/26	9 ± 13	7/10
2 5	29 ± 20	6/6	1 ± 2	2/10
3	13 ± 15	4/6	n.d	n d.
5	1 ± 2	2/12	n.d	n d.
6	5 ± 8	4/4	0 1 ± 0 3	1/10
12	3 ± 5	5/9	0.2 ± 0 4	1/5

[1]percentage SFB positive fields as determined by light microscopic examination of ileal mucosal smears (Means ± SD for 4-26 animals per group)
[2]number of SFB-positive mice/total number of mice
[3]not determined

Table 2: Influence of Social Status on Colonization of SFB s in the Ileum of Male Cpb:SE (Swiss) Mice

housing	social status	n	SFB score[1]	caecal weight[2]	caecal fusi-forms[3]	change of body weight[4]
group	dominant	7	79 ± 19[a]	2.3	85	+ 5
	indifferent	18	64 ± 30[a]	1 9	69	+ 7
	subordinate	11	27 ± 31[b]	2.2	80	- 5
individual	(not applicable)	24	74 ± 23[a]	2.3	80	+ 9

[1]percentage SFB-positive fields as determined by light microscopic examination of ileal mucosal smears (Means ± SD), values not sharing the same superscript letter are significantly different ($p<0.05$; Mann-Whitney U test)
[2]Group mean caecal weight expressed as percentage of body weight.
[3]Group mean number of fusiforms per 100 caecal bacteria as determined by light microscopic examination of Gram-stained smears of caecal contents
[4]Group mean change of body weight expressed as percentage of initial body weight.

DISCUSSION AND CONCLUSION

The results indicate that strain and age are important host factors determining the colonization density of SFBs in the ileum of mice. This is in accordance with previous findings of Koopman *et al.*[5,6,9] and with data on SFBs in chickens as reported by Käufer and Sobiraj.[1] Since colonization of the small intestine by SFBs depends on intimate adherence of this bacterium to epithelial cells,[5] it could be suggested that strain and age influence the characteristics of the mucosa and thereby colonization of SFBs.

A difficultly controllable factor in biomedical research but with great impact on the composition of the intestinal microbiota,[8] is environmental stress.[8] We found that stress in mice caused by aggressive behaviour of counterparts reduces SFB colonization. Similar effects were reported for hyperbaric stress[10] and for stress induced by adverse housing factors.[8] Again, it is attractive to speculate that changes in mucosal properties are responsible for the stress-mediated inhibition of SFB colonization.

We conclude from the present results that differences in strain, age and cageing can be in part responsible for the between-experiment variation in colonization density of SFBs in mice that has been seen frequently.

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2.3 Different degree of ileal colonization by segmented, filamentous bacteria in two strains of mice

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SUMMARY

Segmented, filamentous bacteria (SFBs) are autochthonous, apathogenic inhabitants of the ileum of various animal species. Swiss (Cpb:SE) mice have significantly higher degrees of SFB colonization than do BALB/c mice. The present studies were carried out to identify determinants of this strain difference. In a cross-fostering experiment it was shown that SFB colonization of the pups is determined by the strain of the pups themselves rather than by the strain of the nursing dam. Thus, maternal effects may not be involved in SFB colonization. In a cross-infecting experiment using germ-free and SFB-positive animals of the two strains, it was found that ileal SFB colonization is determined by host characteristics rather than by origin of the SFBs. Thus, strain-specific SFBs may not exist in the two strains of mice. It is concluded that the strain difference in SFB colonization is determined by host characteristics, which probably have a genetic basis.

INTRODUCTION

In the ileum of mice and rats, mucosa-associated, segmented, filamentous bacteria (SFBs) occur that are considered to be autochthonous, apathogenic symbionts (Davis and Savage 1974; Chase and Erlandsen 1976; Koopman *et al.* 1987). It has been suggested that SFBs play a role in host resistance to enteric pathogens (Merrell *et al.* 1979; Garland *et al.* 1982). Progress in research on the nature and significance of SFBs is hampered because there is as yet no *in vitro* technique to culture these bacteria (Davis and Savage 1974; Koopman *et al.* 1988).

Between apparently identical mice within a given experiment, the degree of SFB colonization can vary considerably (Koopman *et al.* 1988; Klaasen *et al.* 1990a, 1991b). This also holds for group mean values of SFB colonization from apparently identical groups of mice in different experiments (Klaasen *et al.* 1991a, 1991b). The basis for this inter-individual and inter-experiment variation of SFB colonization in mice is not known. Possibly, this variation may be indicative of the degree of standardization of animal experimentation (Beynen *et al.* 1991).

One potential source of variation of SFB colonization in mice is the strain used. Under identical environmental conditions, young, mature, Cpb:SE (Swiss) mice are

more densely colonized with SFBs than are BALB/c mice (Koopman *et al.* 1988, 1989; Klaasen *et al.* 1990a, 1991a). This strain difference suggests that the appearance of SFBs has a genetic basis. Genetic host factors influencing the gut microflora have been suggested to operate in mice (Itoh *et al.* 1986) and humans (Van de Merwe *et al.* 1983). However, environmental factors such as maternal effects and/or acquired characteristics could also determine the trait for SFB colonization. In addition, there could be strain-specific SFBs with different potency to colonize the ileum. There is evidence for the existence of host species-specific SFB types. Mouse SFBs may not colonize rat ileum, while rat SFBs may not colonize mouse ileum (Tannock *et al.* 1984; Koopman *et al.* 1984).

In the present study, the above mentioned possibilities to explain the difference in SFB colonization between Swiss and BALB/c mice were put to the test. To assess possible maternal and primary host influence, a cross-fostering experiment was carried out. To assess the possible existence of strain-specific SFB types and the role of acquired host characteristics, a cross-infecting experiment was performed by housing together germ-free and SFB-positive mice of the two strains.

MATERIALS AND METHODS

Cross-fostering experiment

Female, primiparous BALB/c and Swiss mice, aged about 2 mths, were used. They were raised in the breeding colony of the Central Animal Laboratory, Catholic University of Nijmegen, and had a SPF microflora as described elsewhere (Koopman *et al.* 1989). From early pregnancy until the end of the experiment, the mice were kept individually in wire-topped type III macrolon cages (RUCO Metaalindustrie Nederland BV, Valkenswaard, The Netherlands). Bedding material was sterilized, commercial sawdust (Woody-Clean type 3/4, J. Rettenmaier und Söhne, Ellwangen-Holzmühle, Germany). Room temperature was 19-23°C, relative humidity 50-70% and light was on from 06.00-18.00 h. The diet used was a gamma-sterilized, commercial, pelleted diet (RMH-GS, Hope Farms BV, Woerden, The Netherlands), which was supplied *ad libitum*. The mice had free access to sterilized tap water.

Within a 48-h interval, all mice produced their litters. Four days after parturition, all pups were ear-marked and part of the pups of each dam were exchanged with part of the pups of a dam of the other strain. Thus, each BALB/c dam generally nursed half of the offspring of a Swiss dam, and vice versa (Table 1). Contamination with SFBs of the foster pups by their natural dams prior to fostering was considered unlikely because SFBs have never been detected in mice younger than 20 days (Davis and Savage 1974; Koopman *et al.* 1987). During the experimental period, the dams and their pups were not removed from their cage so as to prevent contamination of the pups from sources other than the dam intended. Animal care was performed under strictly clean conditions. The number of pups per cage varied from 4 to 9 (Table 1). At 5½ wks after birth, the pups and dams were killed by cervical dislocation. Body weight, SFB colonization of the ileum, caecum weight, percentage caecal fusiform-shaped bacteria and concentration of faecal Enterobacteriaceae were determined.

Cross-infecting experiment

Female, SPF BALB/c and Swiss mice as described above were used. They were aged 6-8 wks. Female and male germ-free mice of both strains were also used. They were aged 5-8 wks and derived from the germ-free breeding colony of the Central Animal Laboratory, Catholic University of Nijmegen. The germ-free mice had been reared in macrolon type III cages placed in Trexler-type, plastic isolators. Room conditions were as described above. The mice were transferred aseptically into type S macrolon cages (RUCO Metaalindustrie Nederland BV), provided with a filter cap and located in a sterile laminar flow cabinet. Each cage contained two germ-free mice of the same strain; there were six cages per strain. In each of three cages per strain, an SPF mouse of the same strain was placed. In each of the three other cages per strain, an SPF mouse of the other strain was placed. Thus, each cage contained two germ-free recipients and one SPF donor mouse. The SPF mice were ear-marked so as to tell them apart from their germ-free cage mates. Prior to the experiment, the SPF mice were verified to be SFB-positive by checking their litter mates (examination of the small intestine as described below). All mice received a sterilized, pelleted, commercial diet (SRM-A, Hope Farms BV, Woerden, The Netherlands) and sterilized tap water *ad libitum*. Room conditions

were as described above. During the 2-wk experimental period all cages remained closed to prevent contamination of the germ-free mice from sources other than their SPF cage mate. At the end of the experiment all mice were killed by cervical dislocation. Body weight, SFB colonization of the ileum, caecum weight and percentage caecal fusiforms were determined.

Measurements

From the killed mice the small bowel was removed and colonization of its distal half by SFBs was assessed with the use of Gram-stained, mucosal smears as described elsewhere (Klaasen *et al.* 1990b). Colonization of each mouse is expressed as SFB score. This is the mean value of the percentage of SFB-positive fields in 5 smears per animal (light microscopic examination of 20 fields per smear; magnification, 1000x). The incidence of SFB appearance is expressed as the number of SFB-positive animals out of the total number of animals. The caecum with contents was removed, and its weight expressed as percentage of body weight (relative caecal weight). The percentage of caecal fusiform-shaped bacteria (fusiforms) in Gram-stained smears of caecal contents was determined light microscopically, by counting the number of fusiforms per 100 bacteria. To collect faecal samples, the pups of the cross-fostering experiment were placed individually in a clean cage for a few minutes. The concentration of faecal Enterobacteriaceae was determined as described (Koopman *et al.* 1986).

RESULTS

Cross-fostering experiment

At the end of the experiment, i.e. at 5½ wks after parturition, body weights (g) of the dams were as follows: BALB/c mice, 21.7 $\pm$ 0.8 (mean $\pm$ SD, n=5); Swiss mice, 33.0 $\pm$ 3.9 (n=5). Body weights of the pups that suckled with the BALB/c dams were 14.4 $\pm$ 2.3 (n=14) for the natural and 22.3 $\pm$ 3.3 (n=15) for the Swiss young. The natural and foster pups nursed by the Swiss dams weighed 21.8 $\pm$ 4.5 (n=16) and 16.7 $\pm$ 2.0 (n=15), respectively. Apparently, body weights of

the pups were determined by their own strain rather than by the strain of the nursing mouse. Irrespective of the nursing dam, the Swiss pups were significantly heavier than the BALB/c pups ($P<0.05$, Student's *t* test).

SFB scores of the BALB/c and Swiss dams, aged about 4 mths, were not only relatively low, but also comparable: the mean scores were 6 and 3, respectively (Table 1). In contrast, mature, but young Swiss mice have significantly higher SFB scores than their BALB/c counterparts (Koopman *et al.* 1988, 1989; Klaasen *et al.* 1990a, 1991a). The observation here that BALB/c and Swiss dams did not differ as to SFB colonization relates to the fact that ageing is associated with disappearance of SFBs (Klaasen *et al.* 1990a). The origin of the pups had a greater impact on SFB colonization in the pups than the strain of the nursing animal (Table 1). Irrespective of the nursing strain, the Swiss pups had significantly higher SFB scores than the BALB/c pups ($P<0.05$, Mann-Whitney U test). There was no maternal effect on SFB colonization in the pups. Sex of the pups did not influence SFB scores (data not shown), which corresponds to earlier results (Klaasen *et al.* 1990a).

BALB/c mice had significantly higher relative caecal weights than Swiss mice ($P<0.05$, Student's *t* test). Relative caecal weights of the BALB/c and Swiss dams were 1.7 $\pm$ 0.3 % (mean $\pm$ SD, n=5) and 1.2 $\pm$ 0.2 % (n=5). The values for the natural and foster pups were as follows: BALB/c tenders, 1.6 $\pm$ 0.3 % (n=14) and 1.5 $\pm$ 0.3 % (n=15); Swiss tenders, 1.2 $\pm$ 0.3 % (n=16) and 1.5 $\pm$ 0.4 % (n=15). Concerning percentage caecal fusiforms and concentration of faecal Enterobacteriaceae there were neither significant differences ($P>0.05$, Student's *t* test) between BALB/c and Swiss dams, nor between origin and nursing dam of the pups. The percentages caecal fusiforms in the BALB/c and Swiss dams were 44 $\pm$ 15 and 56 $\pm$ 13 %. For natural and foster pups, the values were 56 $\pm$ 13 and 60 $\pm$ 13 % with BALB/c tenders, and 52 $\pm$ 14 and 44 $\pm$ 11 % with Swiss tenders. The numbers of faecal Enterobacteriaceae, expressed as $\log_{10}$ number/g faeces, for natural and foster pups were 1.4 $\pm$ 0.9 and 1.1 $\pm$ 0.5 with BALB/c tenders, and 1.6 $\pm$ 1.1 and 1.5 $\pm$ 1.1 with Swiss tenders.

Cross-infecting experiment

In this experiment, we investigated whether SFB colonization depends on either the bacterial symbiont type or host type. Germ-free BALB/c and Swiss mice were housed together with SFB-positive mice from their own or the other strain. The SPF BALB/c and Swiss SPF donor mice had the following characteristics: mean SFB score, 9 and 35 (n=6); SFB incidence, 6/6 and 6/6; body weight, 20.6 ± 2.0 and 29.0 ± 4.3 g (mean ± SD, n=6); relative caecal weight, 1.7 ± 0.4 and 1.8 ± 0.2 %; caecal fusiforms, 67 ± 19 and 82 ± 11 %. Thus, the Swiss mice had significantly higher ($P<0.05$, Mann-Whitney U test) SFB scores than their BALB/c counterparts, as would be expected (Koopman *et al.* 1988, 1989; Klaasen *et al.* 1990a, 1991a).

After housing together with the SFB donors, all germ-free recipient mice had become SFB-positive (Table 2). However, BALB/c recipients had significantly lower SFB scores ($P<0.05$, Mann-Whitney U test) than the Swiss recipients, irrespective of whether the SFB donors were of the BALB/c or Swiss strain. In fact, the donor strain did not significantly influence SFB scores.

Relative caecal weight and percentage of caecal fusiforms in the recipient mice were not significantly influenced ($P>0.05$, Student's *t* test) by strain of the SFB donor mice. The values for BALB/c recipients with BALB/c and Swiss donors were as follows: relative caecal weight 1.8 ± 0.5 and 2.3 ± 0.5% (mean ± SD, n=6); percentage of caecal fusiforms 63 ± 17 and 64 ± 14%. For Swiss recipients with BALB/c and Swiss donors the values were: relative caecal weight 1.7 ± 0.3 and 2.3 ± 0.8%; percentage of fusiforms 79 ± 10 and 87 ± 5%. Thus, Swiss donors tended to elevate relative caecal weight in the recipients. Swiss recipients had somewhat higher percentages of caecal fusiforms than BALB/c recipients.

DISCUSSION

The present studies were carried out in an attempt to identify determinants of the different degree of SFB colonization in Swiss and BALB/c mice. Young, mature Swiss mice have high degrees of SFB colonization when compared with BALB/c

Table 1. Ileal colonization by segmented, filamentous bacteria (SFBs) in the cross-fostering experiment with SPF BALB/c and Swiss mice

Nursing dams			Natural pups		Foster pups		
Strain	Dam No.	SFB score	Mean SFB score	SFB incidence	Natural dam No.	Mean SFB score	SFB incidence
BALB/c	1	0	11	3/3	8	37	2/3
	2	0	19	4/4	9	27	3/3
	3	9	4	1/2	10	39	2/2
	4	8	39	2/2	6	72	4/4
	5	11	5	3/3	7	54	3/3
Swiss	6	3	32	6/6	4	12	3/3
	7	2	69	3/3	5	23	2/2
	8	4	35	2/2	1	6	3/4
	9	0	47	3/3	2	2	1/3
	10	5	70	2/2	3	18	3/3

Table 2. Ileal colonization by segmented, filamentous bacteria (SFBs) in the cross-infecting experiment with SPF donor and germ-free recipient BALB/c and Swiss mice

Donor mice			Recipient mice		
Strain	Mean SFB score	SFB incidence	Strain	Mean SFB score	SFB incidence
BALB/c	15	3/3	BALB/c	14	6/6
BALB/c	2	3/3	Swiss	42	6/6
Swiss	28	3/3	Swiss	26	6/6
Swiss	43	3/3	BALB/c	8	6/6

mice (Koopman *et al.* 1988, 1989; Klaasen *et al.* 1990a, 1991a). The cross-fostering experiment demonstrated that SFB colonization of the pups was determined by the strain of the pups themselves rather than by the strain of the nursing dam. Swiss pups had high SFB scores, irrespective of whether they were nursed by their natural or by foster dams. This suggests that the trait for SFB colonization is at least partly fixed by characteristics of the host, genetic make-up possibly being important. Maternal effects may not be involved in SFB colonization. However, influences other than genetic factors could be involved. We cannot exclude the possibility that the Swiss pups were already contaminated with SFBs from their natural mother before the age of four days, i.e. prior to the beginning of cross-fostering. Mice younger than 20 days are not colonized by SFBs (Davis and Savage 1974; Koopman *et al.* 1987), but a delayed colonization due to the existence of surviving vegetative SFBs or SFB spores might occur (Chase and Erlandsen 1976; Koopman *et al.* 1988). This could explain the high SFB scores in Swiss pups nursed by BALB/c dams. However, it does certainly not explain the low SFB scores in BALB/c pups nursed by Swiss dams.

A striking observation in the cross-fostering experiment was that in two BALB/c dams and one Swiss dam no SFBs were detected, whereas their natural pups as well as their foster pups were SFB-positive. This implies that these sucklings were infected and colonized by low numbers of SFBs as these SFBs must have been excreted by dams with a very poorly colonized ileum. Thus, the degree of SFB colonization may be independent of the number of ingested SFBs. In an earlier study with mice, it was found that prevention of coprophagy, and thus of oral reingestion of faecal SFBs, did not reduce SFB colonization (Klaasen *et al.* 1990b).

To test the possible existence of strain-specific SFBs, and to further assess the influence of host factors on SFB colonization, the cross-infecting experiment was performed. The results indicate that ileal SFB colonization is determined by host characteristics rather than by origin of the SFBs. In germ-free Swiss mice, SFBs from BALB/c and Swiss mice were equally effective in colonizing the ileum. On the other hand, germ-free BALB/c mice did not become densely colonized, irrespective of whether BALB/c or Swiss mice were used as donors. Thus, it would appear that the observed strain differences in SFB scores were caused by different characteristics of the strains. This corroborates the outcome of the cross-fostering experiment. Since a strong attachment of SFBs to epithelial cells is

essential for colonization of the ileum (Davis and Savage 1974; Chase and Erlandsen 1976; Koopman *et al.* 1987), the strain difference in SFB colonization could relate to epithelial cell surface characteristics.

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2.4 Influence of macronutrients on segmented filamentous bacteria in the small intestine of mice

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SUMMARY

Segmented filamentous bacteria (SFBs) in the ileum of mice were poorly present after the feeding of milk powder as sole source of nutrition, but were abundant in animals fed on a diet based on natural ingredients. Twenty-one different purified diets were formulated so that clues could be obtained to identification of the macronutrients that may be responsible for the differential SFB promoting effects of milk powder and the natural ingredient diet. None of the purified diets induced the appearance of SFBs, suggesting that the type and amount of fat, the type of carbohydrate and the amount and type of fibre in the diet do not influence SFB colonization. Alternatively, the purified diets could have lacked a substance which was present in the natural ingredient diet and is essential for SFB colonization. Thus, although we were not able to identify nutritional factors for SFBs, it is clear that the composition of the diet is at least one factor that determines whether or not SFBs appear in the ileum of mice. Colonization levels of SFBs in mice fed experimental diets were not associated with relative caecal weight, the percentage of fusiform-shaped bacteria in the caecum or the number of Enterobacteriaceae present in faeces. The latter three parameters were however, significantly influenced by the composition of the purified diets.

INTRODUCTION

Segmented filamentous bacteria (SFBs) are intestinal microorganisms that occur strongly associated with epithelial cells in healthy animals of species such as mice, rats, domestic fowl and dogs.[2-6,13-15] The significance of these microorganisms to microecology and colonization resistance of the gastrointestinal tract remains unknown. Progress is hampered by the fact that *in vitro* cultivation of SFBs has so far failed.[6,10,16]

Koopman *et al.*[8,9,11] found inexplicable variations in the presence of SFBs in the ileum of mice which interfere with reproducibility of *in vivo* studies on SFBs. It was clear however, that the composition of the diet is an important determinant of the appearance of SFBs in the small intestine of mice. The feeding of milk powder as sole source of nutrition consistently inhibited the colonization of the ileum by

SFBs when compared with various types of complete laboratory animal diets consisting of natural ingredients.[8,11] This suggests that milk powder either contains substances that inhibit the appearance of SFBs or lacks factors that promote it.

The objective of the present work with mice was to study the influence of macronutrients on the colonization of the ileum by SFBs and on other intestinal parameters. We felt that the difference between milk powder and complete laboratory animal diets, in terms of macronutrients, would be a suitable direction for this work. The macronutrient composition of milk powder is characterized by a high amount of fat rich in saturated fatty acids, lactose as carbohydrate source and lack of fibre. The amount of protein in milk powder, which consists mainly of casein, is similar to that in laboratory animal diets. Thus, purified diets were formulated to study the effect of the amount and type of fat, the type of carbohydrate and the amount and type of fibre on ileal appearance of SFBs in mice.

MATERIALS AND METHODS

Animals and housing conditions

Female Cpb:SE (Swiss) mice, 4 (exp. 1) or 8 wk-old (exp. 2,3,4) and with an SPF flora[12], were used. The animals were kept in macrolon type III cages (RUCO Metaalindustrie Nederland BV, Valkenswaard, The Netherlands), six animals per cage. The bedding material was sawdust, room temperature was 19-23 °C, relative humidity 50-70% and light was on from 06.00-18.00 h. Until the beginning of the experiments, the mice had been fed a commercial pelleted diet (RMH-TM^R, Hope Farms BV, Woerden, The Netherlands) *ad libitum*.

Experimental diets

Four experiments were carried out. In each experiment other than exp. 1 in which two control diets were employed, three identical control diets were used so as to assess the reproducibility of SFB appearance between experiments. There was a negative control diet consisting of milk powder (diet 1), a positive control diet

consisting of natural ingredients (diet 2) and a purified control diet (diet 3). Earlier work[8,11] had shown that milk powder inhibits SFB colonization. Separate batches of control diets were used for each experiment. The diets were stored at 4°C until use.

The first experiment had seven dietary groups (diet 2 was not used) and the other three had eight dietary groups, each group consisting of six mice. Table 1 shows the composition of the experimental diets. In exp. 1, the amount and type of fat, either corn oil or coconut fat, were the dietary variables. Extra fat was incorporated into the diet at the expense of isocaloric amounts of corn starch and dextrose in a 1:1 ratio (w/w). Exp. 2 compared the effects of galactose, glucose, fructose, sucrose and lactose. The effects of tallow, cocoa fat, butter fat, sunflowerseed oil and olive oil were compared in exp. 3. In exp. 4, the diets differed in the amount and type of fibre (cellulose versus pectin). All diets were in pelleted form, except for the diets containing the various carbohydrates (exp. 2), which were in meal form. Diet 1 (milk powder, Vremimel[R]; Klaver BV, Hoofddorp, The Netherlands) was mixed with water, dried and supplied as 2-4 mm-thick fragments of 1-2 cm^2. The animals had free access to the diets and tap water. Each experiment lasted 30 days.

Parameters measured

The animals were weighed at the beginning of each experiment and again at the end of each experiment, following death by cervical dislocation. The SFB score (colonization density of SFBs) of the distal half of the small intestine was measured by preparing and scoring Gram-stained smears of the mucosa, as described by Klaasen *et al.*[5] The SFB score of each mouse was the percentage of SFB-positive fields (magnitude 1000x) in five small intestine sections (numbered distalwards from five to nine). The caeca with contents were removed, weighed and expressed as percentage of body weight (relative caecal weight, RCW). The percentage of caecal fusiform-shaped bacteria ('fusiforms') in samples of 100 bacteria in Gram-stained smears of caecal contents was determined. Individual faecal samples were collected from mice after they had been placed individually in a cage for a few minutes. The concentration of faecal Enterobacteriaceae was determined according to Koopman *et al.*[7] The RCW, the percentage of caecal fusiforms and the

Table 1 Composition of the experimental diets (diets 1 and 2 are described in the notes below)

Ingredient	Diet composition (g/1000 g)					
	3*	4	5	6	7	8
Experiment 1						
Corn oil	25 00	50 00	100 00	25 00	200 00	25 00
Coconut fat	25 00	—	—	75 00	—	175 00
Corn starch	326 30	326 30	270 05	270 05	157 55	157 55
Dextrose	326 30	326 30	270 05	270 05	157 55	157 55
Salts, vitamins	66 40	66 40	66 40	66 40	66 40	66 40
Constant components†	231 00	231 00	231 00	231 00	231 00	231 00
Total	1000 00	1000 00	937 50	937 50	812 50	812 50
Experiment 2						
Corn starch	326 30	—	—	—	—	—
Dextrose	326 30	—	—	—	—	—
Galactose	—	652 60	—	—	—	—
Glucose	—	—	652 60	—		—
Fructose	—	—	—	652 60	—	—
Sucrose	—	—	—	—	652 60	—
Lactose	—	—	—	—	—	652 60
Salts, vitamins	66 40	66 40	66 40	66 40	66 40	66 40
Constant components‡	281 00	281 00	281 00	281 00	281 00	281 00
Total	1000 00	1000 00	1000 00	1000 00	1000 00	1000 00
Experiment 3						
Corn oil	25 00	—	—	—	—	—
Coconut fat	25 00	—	—	—	—	—
Tallow	—	50 00	—	—	—	—
Cocoa fat	—	—	50 00	—	—	—
Butter fat	—	—	—	50 00	—	—
Sunflowerseed oil	—	—	—		50 00	—
Olive oil	—	—	—	—	—	50 00
Salts, vitamins	66 40	66 40	66 40	66 40	66 40	66 40
Constant components§	883 60	883 60	883 60	883 60	883 60	883 60
Total	1000 00	1000 00	1000 00	1000 00	1000 00	1000 00
Experiment 4						
Corn starch	326 30	311 30	281 30	326 30	311 30	281 30
Dextrose	326 30	311 30	281 30	326 30	311 30	281 30
Cellulose	30 00	60 00	120 00	—	—	—
Pectin	—	—	—	30 00	60 00	120 00
Salts, vitamins	66 40	66 40	66 40	66 40	66 40	66 40
Constant components‖	251 00	251 00	251 00	251 00	251 00	251 00
Total	1000 00	1000 00	1000 00	1000 00	1000 00	1000 00

Composition of *diet 1* (milk powder, negative control diet), which was identical for all experiments (g/kg dry matter) casein, 269, fat, 249, lactose, 415, salts and vitamins, 67 (data provided by the manufacturer)

Composition of *diet 2* (natural ingredient diet, positive control diet), which was identical for all experiments (g/kg diet) skim milk powder, 145, lucern meal 50, native corn starch, 99, soybean oil, 20, ground barley, 300, fish meal, 50, soybean protein concentrate (48 8 per cent of crude protein), 80, wheat middlings, 100, corn protein concentrate (60 per cent of crude protein), 78, molasses, 60, vitamin premix, 6, mineral premix, 5, calcium carbonate, 4, sodium chloride, 3 Calculated concentrations of nutrients (g/100 g) crude protein 23, crude fat, 4 5, crude fibre, 3 5, calcium, 0 65, phosphorus, 0 55

*The composition of *diet 3* was identical for all experiments

†Composition (g) casein, 151, molasses, 50, cellulose, 30

‡Composition (g) casein, 151, corn oil, 25, coconut fat, 25, molasses, 50, cellulose, 30

§Composition (g) casein, 151, corn starch, 326 3, dextrose, 326 3, molasses, 50, cellulose, 30

‖Composition (g) casein, 151, corn oil, 25, coconut fat, 25, molasses, 50

The salts and vitamins consisted of (g) $CaCO_3$, 12 4, $NaH_2PO_4 2H_2O$, 15 1, Na_2CO_3, 6 8, $MgCO_3$, 1 4, KCl, 1 0, $KHCO_3$ 7 7, mineral premix, 10 0, vitamin premix, 12 0

The composition of the vitamin and mineral premixes (used in diets 2–8) have been described [1]

concentration of faecal Enterobacteriaceae were determined as general indicators for the quality of the intestinal flora and to study possible correlations with SFB colonization.

Statistical analysis

The Mann-Whitney U test was used to compare diet groups 1 and 2 within each experiment. The parameter-free Kruskal-Wallis rank statistics test was used to detect significant treatment effects among diet groups 3-8 within each experiment. A maximum probability of 0.05 for the type I error was used.

RESULTS AND DISCUSSION

In exp. 1, using younger mice, body weights increased on average by 62 g; there were no effects of the composition of the diet. In exp. 2-4, body weights increased on average by 24 g. Apart from the diet containing galactose (diet 4, Exp. 2), which actually produced a body weight loss of 11% compared with the initial value, there were no dietary effects on weight gain during the course of the experiments (results not shown).

Table 2 shows that the negative and positive control diets (diets 1 and 2) systematically produced significantly different values for SFB colonization. In mice fed the milk powder diet (diet 1), SFB scores were lower than in mice fed the natural ingredient diet (diet 2). The purified diets were formulated so that the effects of a single component could be studied. However, none of the purified diets produced appreciable SFB colonization, and among the diets there were no significant differences. Thus, this study suggests that the amount and type of fat, the type of carbohydrate and the amount and type of fibre in the diet do not influence SFB appearance in the ileum of mice. On the other hand, it could be suggested that the purified diets and the milk powder diet, in contrast to the natural ingredient diet, lacked a substance that is essential for colonization by SFBs.

High RCWs (above 2%) were seen in the mice fed milk powder and those fed the purified diets containing galactose (diet 4, exp. 2) or lactose (diet 8, exp. 2). Thus, caecal weights were significantly increased after the feeding of galactose

either as galactose or in the form of lactose. Significantly reduced percentages of caecal fusiforms were observed in mice fed milk powder or the purified diets containing either galactose (diet 4, exp. 2), lactose (diet 8, exp. 2) or a high amount of pectin (diet 8, exp. 4). Feeding milk powder increased the number of faecal Enterobacteriaceae. It was also significantly affected by the experimental diets in exp. 2-4, but the differences among dietary groups were relatively small. Thus, SFB scores in mice fed the experimental diets did not correlate with RCW, the percentage of fusiforms in the caecum or the number of Enterobacteriaceae present in faeces.

The main conclusion from these experiments is that, although we have not been able to identify essential nutritional factors that enhance colonization by SFBs, diet does clearly influence SFB colonization in mice. Further studies will focus on the isolation of the SFB promoting substances in the natural ingredient diet.

Table 2 Mean values* of SFB score, relative caecal weight, percentage caecal fusiforms and faecal *Enterobacteriaceae* in mice fed various diets

Experiment	Diet†								Significance§
	1‡	2	3	4	5	6	7	8	
	SFB score (percentage SFB-positive fields)								
1	1 2	—	1 0	0 0	0 2	0 0	0 3	0 0	NS
2	0 2	13 0	0 0	7 2	0 0	0 0	0 0	0 0	NS
3	0 0	29 2	0 3	0 5	0 7	0 2	0 0	0 0	NS
4	0 7	30 5	0 0	2 2	1 2	0 2	0 2	0 0	NS
	Relative caecal weight (g/100 g body weight)								
1	3 5	—	0 6	0 6	0 4	0 7	0 5	0 5	NS
2	3 2	1 4	0 6	3 4	0 6	0 5	0 5	4 5	S
3	3 4	2 0	0 7	0 7	0 8	0 6	0 7	0 7	NS
4	3 1	2 0	0 6	0 7	0 7	0 8	1 0	1 4	S
	Percentage caecal fusiforms (number/100 caecal bacteria)								
1	52	—	95	96	82	100	99	100	NS
2	10	93	80	14	93	100	77	0	S
3	23	85	79	69	78	74	78	76	NS
4	1	67	76	81	89	70	44	14	S
	Faecal *Enterobacteriaceae* ($\log_{10}$ number/g faeces)								
1	5 0	—	4 0	5 3	4 7	5 0	4 3	4 7	NS
2	7 0	5 7	5 3	9 2	4 8	5 2	5 2	7 2	S
3	8 3	4 3	6 0	5 5	6 7	4 0	3 7	4 2	S
4	6 8	3 5	3 2	4 3	4 0	2 7	5 3	6 3	S

*Mean values for six mice per dietary group
†The composition of the diets is given in Table 1
‡Values of diet groups 1 and 2 in the same row are significantly different ($P < 0.05$, Mann–Whitney U test)
§Significance of treatment among diet groups 3–8: probability of type I error: NS, $P > 0.05$; S, $P < 0.05$ (Kruskal–Wallis rank statistics test)

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2.5 Influence of diets containing native or boiled *Phaseolus vulgaris* on segmented filamentous bacteria in the small intestine of mice

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SUMMARY

The hypothesis was tested that the appearance of segmented filamentous bacteria (SFBs) in the small intestine of mice is inhibited by dietary lectins. Mice were fed diets containing either native or boiled *Phaseolus vulgaris*. Boiling denaturated the lectins in *Phaseolus*. Not only was the hypothesis disproved, it was found that native, when compared with boiled *Phaseolus*, consistently stimulated SFB colonization.

INTRODUCTION

Colonization of the small intestine by segmented filamentous bacteria (SFBs) depends on intimate adherence of these bacteria to the mucosa.[1] Thus, it could be hypothesized that compounds that reduce the structural integrity of the mucosa should interfere with the colonization of SFBs. Red kidney beans (*Phaseolus vulgaris* 'Processor') contain the lectin phytohaemagglutinin (PHA), a glycoprotein which reduces growth[9] and causes damage to the small intestinal mucosa in rats.[3] The mucosa damaging activity of PHA in *Phaseolus vulgaris* can be destroyed by boiling of the beans.[3] Consequently, our hypothesis implies that SFB appearance in the small intestine of mice is inhibited after the feeding of a diet containing native *Phaseolus vulgaris*, when compared with a diet containing boiled *Phaseolus vulgaris*. In the present study this hypothesis was tested.

MATERIALS AND METHODS

Female, 6 wk-old BALB/c mice and 7 wk-old Cpb:SE (Swiss) mice, both with a specified pathogen free (SPF) flora[8] , were used. They had been fed a commercial, pelleted diet (RMH-TM^{R}, Hope Farms BV, Woerden, The Netherlands). The mice were kept, five animals per cage, in wire-topped macrolon type III cages (RUCO Metaalindustrie Nederland BV, Valkenswaard, The Netherlands), with sawdust as bedding material. Room temperature was 19-23°C, relative humidity 50-70%, and light was on from 06.00-18.00 h.

Three experiments of identical design were carried out. Each experiment had four dietary groups consisting of 10 mice each. In Expt 1, BALB/c mice were used, and in Expts 2 and 3 Cpb:SE mice. At the beginning of the experiments, mean body weights were 14.1 (BALB/c, Expt 1), 24.3 (Cpb:SE, Expt 2) and 26.1 (Cpb:SE, Expt 3) g.

One dietary group of each experiment was fed milk powder as sole source of nutrition and served as a negative control group in which SFB colonization should be blocked.[6] There was a positive control group which was fed the natural ingredient diet which produces SFB colonization consistently.[6] The other two groups of each experiment were fed the test diets: natural ingredient diets containing either native or boiled *Phaseolus vulgaris*. All mice were fed the positive control diet for one week. Then, they were divided into four dietary groups. One group remained on the positive control diet. The other animals were transferred either to the negative control diet or the test diets. The experimental period lasted 30 days. The mice were killed by cervical dislocation.

The milk powder diet was prepared as described.[6] The composition of the pelleted natural ingredient diet can be found elsewhere.[6] To formulate the pelleted test diets, 200 g/kg of either native or boiled *Phaseolus vulgaris* 'Processor' was added to the positive control diet at the expense of part of the skim milk powder (50 g/kg), ground barley (100 g/kg) and wheat middlings (50 g/kg) components. The boiled beans were prepared by boiling in tap water for 30 min, drying and grinding. In Expts 1 and 2, the same diet batches were used. For Expt 3, separate batches of diet were prepared. Diets were stored at 4°C until use. In each experiment, the animals had free access to feed and tap water.

To check whether *Phaseolus* lectins were indeed denaturated by boiling, the concentration of lectins in native and boiled *Phaseolus*, as well as in the complete diets, were determined by an ELISA method.[2] With this ELISA method, the five isolectins in *Phaseolus*, namely E4, E3L, E2L2, EL3 and L4, were detected.

The determination of the level of SFB colonization and other intestinal parameters has been described elsewhere.[5,7]

RESULTS AND DISCUSSION

Table 1 shows that boiling of *Phaseolus* drastically reduced total lectin concentrations. The composition of the diet did not affect final body weights (Table 2). No clinical abnormalities were seen in any of the dietary groups. Examination of the small intestine, caecum and large bowel did not reveal any macroscopically visible lesions. Thus, it can be concluded that the mice fed the experimental diets were apparently healthy.

Table 1 Lectin concentrations (μg/g product) in native and boiled *Phaseolus vulgaris* and in experimental diets

Product	Experiments 1 and 2	Experiment 3
Native *Phaseolus*	39 000	52 500
Boiled *Phaseolus*	645	270
Diet without *Phaseolus*	10	3
Diet with native *Phaseolus*	7 350	5 525
Diet with boiled *Phaseolus*	82	112

Phaseolus vulgaris is rich in phytohaemagglutinin, a lectin which, in purified form, is known to damage the mucosa in rats[3] and reduce growth performance.[9] Apparently, mice are rather insensitive to the feeding of diets containing native *Phaseolus*.

Irrespective of the composition of the diet, BALB/c mice (Expt 1) displayed markedly lower SFB scores than Swiss mice (Table 2) which is in agreement with the results of another experiment.[4] This suggests that genetic characteristics of the host interact with the microecology of the gastrointestinal tract. Perhaps, genetically determined characteristics of the structure of the intestinal mucosal surface are involved, because SFB colonization is invariably associated with bacterial attachment to the mucosa.[1] In the Swiss mice, a statistically significant increase in SFB score was observed after feeding the positive control diet, when compared with milk powder (Table 2). This corroborates our earlier work with this strain of mice.[6] The diet containing native *Phaseolus* produced consistently higher group mean SFB scores than the diet containing boiled *Phaseolus*. This outcome is completely opposite to what we expected on the basis of our hypothesis. The fact

that addition to the positive control diet of both native and boiled *Phaseolus* stimulated SFB colonization, suggests that dietary lectins do not influence SFBs. Native *Phaseolus* in the diet induced higher relative caecal weights than boiled *Phaseolus* but overall there was no relation between caecal weight and SFB colonization. Native and boiled *Phaseolus* in the diet of Swiss mice did not differently influence percentage caecal fusiforms and the number of Enterobacteriaceae in faeces (Table 2).

Table 2 Body weights and microecological parameters of the intestine of mice fed the experimental diets[1]

Parameter	Experiment[2]	Milk powder	Control diet	Diet with native *Phaseolus*	Diet with boiled *Phaseolus*
Final body weight (g)	1	17 1 (1 0)	15 6 (2 2)	15 4 (1 5)	17 0 (1 8)
	2	27 5 (2 6)	25 7 (3 3)	27 4 (2 7)	26 9 (3 2)
	3	29 3 (3 2)	27 8 (2 3)	27 2 (2 6)	27 2 (2 2)
SFB score[3]	1	0 0	0 6	1 0	0 0
	2	0 1	9 4*	42 3	29 1
	3	2 2	9 5*	21 6	11 5
Number of SFB-positive animals	1	0 10	1 10	2/10	0/10
	2	1 10	6 10*	10/10	10/10
	3	5 10	9 10*	9/9	8/10
Relative caecal weight*	1	2 4 (0 3)	2 3 (0 4)	3 9 (0 7)	2 6 (0 2)†
	2	2 4 (0 4)	1 9 (0 4)*	2 8 (0 4)	2 4 (0 4)
	3	2 9 (0 6)	2 5 (0 4)	4 1 (1 0)	2 7 (0 4)†
Percentage caecal fusiforms[5]	1	55 6	68 6	65 0	75 4
	2	32 4	74 5*	77 8	79 4
	3	56 1	87 2*	68 9	80 8
Faecal Enterobacteriaceae[6]	1	5 7	4 3	4 3	2 3†
	2	6 8	5 1*	3 7	4 0
	3	7 3	4 3*	6 0	5 2

[1]Means and standard deviations (in parentheses), incidences or means (not normally distributed data) are given for 10 animals per dietary group
[2]In experiment 1 BALB c mice were used, in experiments 2 and 3 Cpb SE (Swiss) mice were used
[3]Percentage of SFB-positive fields (magnitude × 1000)
[4]Gram 100 g body weight
[5]Number 100 caecal bacteria
[6]Log_{10} number g faeces
*Significantly different ($P<0.05$ Student's t or Mann–Whitney U test) from mice fed milk powder
†Significantly different ($P<0.05$ Student's t or Mann–Whitney U test) from mice fed the diet with native *Phaseolus*

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2.6 Influence of a natural-ingredient diet containing *Phaseolus vulgaris* on the colonization by segmented, filamentous bacteria of the small bowel of mice

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SUMMARY

The appearance of segmented, filamentous bacteria (SFBs) in the small bowel of mice is influenced by the composition of the diet, but the dietary components responsible are not known. The addition of ground, whole *Phaseolus vulgaris* to a natural-ingredient diet at the expense of part of the skim milk powder, ground barley and wheat middlings components, caused an increase of the colonization of the mouse small bowel by SFBs. This effect was not seen when whole *Phaseolus* was added to a purified diet at the expense of part of the casein, corn oil, coconut fat, corn starch, dextrose and cellulose components. In an attempt to identify the fraction of *Phaseolus* that might contain SFB-inducing substances, the skin and kernel fraction of the bean were added to the natural-ingredient diet. The skin and kernel fraction were found to be as effective in inducing SFB appearance as was whole *Phaseolus*.

INTRODUCTION

Segmented, filamentous bacteria (SFBs) are non-cultivable, apathogenic bacteria inhabiting the distal small intestine of mice and rats [1-3]. SFBs could contribute to host resistance to infections [4]. Colonization of the mouse intestine by SFBs is influenced by the composition of the diet [5-7]. In a preliminary study, the addition of native *Phaseolus vulgaris* to the natural-ingredient diet of mice stimulated colonization of the small bowel by SFBs [8]. The objectives of the present feeding experiments with mice were as follows. First, we wanted to check the reproducibility of the earlier observed stimulatory effect of native *Phaseolus* on SFB colonization. Secondly, we addressed the question whether the addition of *Phaseolus* to a purified diet would also stimulate SFB colonization. Previous work indicates that purified diets lack a factor necessary for SFB appearance [5]. Thirdly, an attempt was made to locate the putative SFB-inducing factor in *Phaseolus* by comparing the effects of the kernel and skin of the bean.

MATERIALS AND METHODS

Animals and housing

Two separate experiments were conducted; the interval between them was about nine months. Each experiment lasted four weeks. Female, 7-wk old Cpb:SE (Swiss) mice with a specified pathogen-free (SPF) flora [9] were used throughout. They had been fed a commercial, non-purified, pelleted diet (RMH-TM, Hope Farms BV, Woerden, The Netherlands). During the experimental periods, the mice were kept in wire-topped macrolon type III cages (RUCO Metaalindustrie Nederland BV, Valkenswaard, The Netherlands), with sawdust as bedding material. Room temperature was 19-23°C, relative humidity 50-70%, and light was on from 06.00-18.00 h. The mice had free access to the experimental diets and tap water. For each experiment, separate batches of diet were prepared.

Experiment 1

The animals were divided into five dietary groups. The ingredient composition of the diets is described in Table 1, and the analyzed composition in Table 2. There was a negative control group fed milk powder as sole source of nutrition. The milk powder was prepared and supplied in the form of chunks as described [5]. The composition (g/kg dry matter) was: casein, 269; fat, 249; lactose, 415; salts and vitamins, 67 (data provided by the manufacturer). In the mice fed milk powder, SFB colonization should be negligible [5, 8, 10, 11]. The other four diets were in pelleted form (diameter pellets, 10 mm). The positive control group received a natural-ingredient diet that consistently produces SFB colonization [5, 8]. Ground, native *Phaseolus vulgaris* 'Processor' was added to the natural-ingredient diet at the expense of part of the skim milk powder, ground barley and wheat middlings components (Table 1). We also used a purified diet that essentially prevents SFB colonization [5]. *Phaseolus* was added to the purified diet at the expense of part of the carbohydrate, protein, fat and fibre components (Table 1). These macronutrients had been shown not to influence SFB colonization [5].

Table 1. Ingredient composition of diets used in experiment 1

Natural-ingredient diets				Purified diets	
Addition:	None	*Phaseolus*		None	*Phaseolus*
Ingredient (g)			Ingredient (g)		
Skim milk powder	145.0	95.0	Casein	151.0	106.0
Lucern meal	50.0	50.0	Corn oil	25.0	23.0
Native corn starch	99.0	99.0	Coconut fat	25.0	23.0
Soybean oil	20.0	20.0	Corn starch	326.3	257.8
Ground barley	300.0	200.0	Dextrose	326.3	257.8
Fish meal	50.0	50.0	Molasses	50.0	50.0
Soy protein concentrate	80.0	80.0	Cellulose	30.0	16.0
Wheat middlings	100.0	50.0	$CaCO_3$	12.4	12.4
Corn protein concentrate	78.0	78.0	$NaH_2PO_4.2H_2O$	15.1	15.1
Molasses	60.0	60.0	Na_2CO_3	6.8	6.8
Vitamin premix[1]	6.0	6.0	$MgCO_3$	1.4	1.4
Mineral premix[1]	5.0	5.0	KCl	1.0	1.0
$CaCO_3$	4.0	4.0	$KHCO_3$	7.7	7.7
NaCl	3.0	3.0	Vitamin premix[1]	12.0	12.0
Phaseolus vulgaris[2]	-	200.0	Mineral premix[1]	10.0	10.0
			Phaseolus vulgaris[2]	-	200.0
Total	1000.0	1000.0		1000.0	1000.0

[1] Compositions have been described elsewhere [19].
[2] Ground, native beans.

Experiment 2

There were four diet groups. The ingredient composition of the control natural-ingredient diet was identical to that used in experiment 1. To this diet whole native *Phaseolus* was added, or instead, either the skin or kernel fraction of the *Phaseolus* bean was added. The addition of ground, whole *Phaseolus* was at the level of 200 g/kg diet and at the expense of the same ingredient amounts as described for experiment 1. Beans were broken with the use of the Variostoel apparatus (Miag, Braunschweig, Germany) and skin and kernel separated with the use of the Petkrüs apparatus (Röber, Minden, Germany). On a weight basis, the beans consisted of

12.7% of skin and 87.3% of kernel. The skin fraction was added to the control diet at a level of 25.4 g/kg diet, at the expense of 6.35 g skim milk powder, 12.7 g ground barley and 6.35 g wheat middlings per kg diet. The kernel fraction was added to the control diet at a level of 174.6 g/kg, at the expense of 43.65 g skim milk powder, 87.3 g ground barley and 43.65 g wheat middlings per kg diet. Tables 3 and 4 show the analyzed composition of the bean fractions and the experimental diets.

Table 2. Analyzed composition of diets used in experiment 1

		Natural-ingredient diets[1]		Purified diets[1]	
	Milk powder	None	*Phaseolus*	None	*Phaseolus*
Component (g/100 g)					
Dry matter	92.8	89.7	89.7	90.9	90.6
Nitrogen	4.3	3.6	3.6	2.3	2.2
Fat	17.3	4.1	3.8	5.2	5.0
Ash	5.7	5.4	5.4	4.1	4.8
Calcium	0.76	0.46	0.44	0.44	0.54
Phosphorus	0.77	0.52	0.49	0.41	0.43
Magnesium	0.08	0.13	0.13	0.05	0.08
Fatty acid (g/100 g fatty acids)[2]					
C 12:0	3.9	0.2	0.1	21.9	19.8
C 14:0	11.2	1.1	1.0	9.2	8.4
C 16:0	29.1	14.4	13.5	10.3	11.0
C 18:0	11.9	3.4	3.3	2.7	2.7
C 18:1	26.5	19.9	20.1	17.9	18.6
C 18:2	1.2	44.5	43.7	30.1	30.6
C 18:3 (n-3)	0.6	4.4	7.0	0.5	2.1

[1] Additions are indicated.
[2] Selected fatty acids in shorthand notation.

Table 3. Analyzed composition of native, whole *Phaseolus vulgaris* 'Processor' beans and that of the skin and kernel fractions used in experiment 2

	Whole beans	Skin fraction	Kernel fraction
Component (g/100 g)			
Dry matter	89.8	91.7	90.8
Nitrogen	2.7	1.1	3.0
Fat	1.2	0.5	1.2
Total fibre	38.1	70.5	34.1
Ash	4.2	5.7	4.0
Calcium	0.19	0.99	0.05
Phosphorus	0.42	0.11	0.46
Magnesium	0.17	0.27	0.15
Fatty acid (g/100 g fatty acids)[1]			
C 16:0	11.4	10.7	10.5
C 18:1	14.1	12.6	11.8
C 18:2	29.0	24.2	26.2
C 18:3 (n-3)	38.9	41.7	45.3

[1] Selected fatty acids in shorthand notation.

Measurements

The animals were weighed at the beginning and end of each experiment. They were inspected daily for diarrhoea. Feed intake per cage was assessed by weighing the amount supplied and the remnants in the food hopper.

At the beginning and end of each experiment, individual SFB scores and incidences of SFB-positive animals were determined. The mice were killed by cervical dislocation; the distal half of the small bowel was removed and Gram-stained mucosal smears prepared as described [12]. The degree of colonization is expressed as the SFB score, that is the mean value of the percentage of SFB-positive fields in five smears per animal as examined by light microscopy (20 fields per smear; magnification, 1000x). SFB incidence was the number of SFB-positive animals out of the total. The small bowel was also examined for macroscopic lesions.

Table 4. Analyzed composition of diets used in experiment 2

	Natural-ingredient diets[1]			
	None	Whole beans	Skin fraction	Kernel fraction
Component (g/100 g)				
Dry matter	88.0	87.1	87.9	87.9
Nitrogen	3.6	3.6	3.5	3.7
Fat	4.5	4.4	4.5	4.2
Ash	5.6	5.4	5.6	5.5
Calcium	0.46	0.44	0.47	0.42
Phosphorus	0.55	0.51	0.55	0.52
Magnesium	0.14	0.14	0.14	0.14
Fatty acid (g/100 g fatty acids)[2]				
C 16:0	16.5	16.6	17.3	14.0
C 18:0	3.8	4.0	4.1	3.4
C 18:1	16.9	15.7	15.2	19.6
C 18:2	34.3	30.0	29.5	42.7
C 18:3 (n-3)	3.7	4.4	3.1	6.9

[1,2] See legend to Table 2.

In experiment 1, from a random sample of three animals per diet group, part of the ileum was used for scanning electron microscopy. A piece of ileal tissue (ca. 1 cm^2), located 4 cm proximally to the ileocaecal junction, was excised and flushed with sterile saline. The tissue samples were then fixed at 20°C for 24 h in 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer solution (pH 7.4, 320 Osmol). Subsequently, they were processed and scanning electron micrographs taken as described [1]. Any damage to the intestinal epithelium and the presence of SFBs at the ileal surface were recorded.

The caecum with contents was removed, weighed and the result expressed as percentage of body weight (relative caecum weight). The percentage of caecal fusiform-shaped bacteria in Gram-stained smears of caecal contents was determined light microscopically by counting the number of fusiforms per 100 bacteria. In

experiment 1, just before killing, the mice were placed individually in a novel cage for a few minutes to collect faecal samples. The concentration of faecal Enterobacteriaceae was determined as described [13]. The methods of feed analyses can be found elsewhere [14].

Statistical analyses

A priori defined contrasts were evaluated for statistically significant differences with a one-sided P-value preset at 0.05. In experiment 1, the following dietary groups were compared: group fed milk powder versus group fed natural-ingredient diet; groups fed the natural-ingredient diet without or with *Phaseolus*; groups fed the purified diet without or with *Phaseolus*. In experiment 2, the following dietary groups were compared: groups fed the diets without or with whole beans; groups fed the diets with whole beans or with skin fraction; groups fed the diets with whole beans or with kernel fraction. SFB scores and incidences were compared with Mann-Whitney U test; the other parameters were compared using Student's *t* test.

RESULTS

At the beginning of experiments 1 and 2 the following results were obtained: SFB score (mean and range, n=6), 42 (11-69) and 55 (28-85); SFB incidence, 6/6 and 6/6; relative caecum weight (% of body weight, mean ± SD, n=6), 1.6 ± 0.4 and 1.7 ± 0.2; percentage caecal fusiforms (mean ± SD, n=6), 90 ± 9 and 94 ± 2. All animals were free from macroscopically visible lesions in the small intestine.

In experiment 1, group mean body weights after 4 wks had increased 1 to 3 g (Table 5). None of the animals had diarrhoea during the experiment. The intake of milk powder was significantly lower than that of the natural-ingredient diet without *Phaseolus* (Table 5). This is caused by the higher caloric density of the milk powder (cf. Table 2). In experiment 2, final body weights were somewhat depressed after feeding the kernel fraction (Table 6). Feed intake did not differ between the four groups. Again, none of the animals showed diarrhoea and macroscopic examination of the small intestine revealed no pathologic changes.

Table 5. Body weights and feed intake in experiment 1.[1]

		Natural-ingredient diets[2]		Purified diets[2]	
	Milk powder	None	*Phaseolus*	None	*Phaseolus*
n	9	21	21	9	20
Body weight (g)					
Initial	23.2 ± 2.5	24.5 ± 2.3	22.9 ± 5.4	24.7 ± 2.9	23.9 ± 1.8
Final	26.3 ± 3.2	26.5 ± 3.1	25.0 ± 3.2	27.2 ± 4.2	25.0 ± 1.8
Feed intake (g/cage.28d)	197 ± 16[a]	286 ± 18[a]	266 ± 24	247 ± 12[b]	276 ± 15[b]

[1] Means ± SD. Feed intake is given per cage each containing three mice. Values sharing the same superscript letter are significantly different.
[2] Additions are indicated.

Table 6. Body weights and feed intake in experiment 2.[1]

	Natural-ingredient diets[2]			
	None	Whole beans	Skin fraction	Kernel fraction
n	9	21	21	21
Body weight (g)				
Initial	24.1 ± 2.9	24.7 ± 2.1	24.4 ± 2.8	23.8 ± 1.6
Final	31.0 ± 2.1	29.2 ± 3.3[a]	30.4 ± 3.7	27.5 ± 2.5[a]
Feed intake (g/cage.28d)	337 ± 24	352 ± 24	349 ± 19	339 ± 11

[1,2] See legend to Table 5.

As expected, none of the mice fed either milk powder or the purified diet without *Phaseolus* had SFBs in the ileum (Table 7). Mean SFB scores in mice fed either of the two natural-ingredient diets were relatively low. Nevertheless, the natural ingredient diet without *Phaseolus* produced a significantly higher SFB score and incidence than the milk powder diet. Addition of *Phaseolus* to the natural-

Table 7. Intestinal parameters in mice fed different diets in experiment 1.[1]

		Natural-ingredient diets[2]		Purified diets[2]	
	Milk powder	None	*Phaseolus*	None	*Phaseolus*
n	9	21	21	9	20
SFB score[3]					
Mean	0[a]	2[a,b]	7[b]	0	0.3
Range	-	0-31	0-31	-	0-6
SFB incidence					
LM[4]	0/9[a]	6/21[a]	12/21	0/9	2/20
SEM[5]	0/3	1/3	3/3	0/3	0/3
RCW[6]	2.3 ± 0.7[a]	1.6 ± 0.3[a,b]	2.1 ± 0.4[b]	0.7 ± 0.1[c]	1.6 ± 0.3[c]
Caecal fusiforms (%)	65 ± 14[a]	86 ± 7[a,b]	79 ± 10[b]	85 ± 11[c]	69 ± 14[c]
Faecal Enterobacteriaceae[7]	5.4 ± 0.9[a]	3.9 ± 0.9[a]	4.0 ± 1.0	4.9 ± 0.3	4.8 ± 1.2

[1] Means ± SD; for SFB scores mean values and range are given. Values sharing the same superscript letter are significantly different.
[2] Additions are indicated.
[3] Percentage SFB-positive fields in ileal smears (light microscopy; magnification, 1000x).
[4] LM = light microscopy.
[5] SEM = scanning electron microscopy.
[6] RCW = relative caecum weight (% body weight).
[7] Log_{10} number/g faeces.

ingredient diet, but not to the purified diet, significantly raised the SFB score. In none of the diet groups, scanning electron microscopy of the ileal mucosa showed any lesions in the epithelium. Scanning electron micrographs of SFBs attached to the ileal mucosa of a mouse fed the natural-ingredient diet either with or without *Phaseolus*, are shown in Fig. 1.

In mice fed the natural-ingredient diet without *Phaseolus* versus the milk powder diet, relative caecum weight was significantly lowered, percentage caecal fusiforms was raised and faecal Enterobacteriaceae were depressed (Table 7). This corroborates our earlier experiments [5, 8]. Enrichment of either the natural-ingredient or purified diet with *Phaseolus* significantly elevated relative caecum weight and lowered the percentage of caecal fusiforms. This corresponds to findings described elsewhere [8]. *Phaseolus* consumption did not influence the number of faecal Enterobacteriaceae (Table 7).

Table 8. Intestinal parameters of mice fed different diets in experiment 2.[1]

	Natural-ingredient diets[2]			
	None	Whole beans	Skin fraction	Kernel fraction
n	9	21	21	21
SFB score[3]				
Mean	2[a]	12[a]	10	11
Range	0-5	0-36	0-61	0-41
SFB incidence (light microscopy)	4/9[a]	19/21[a]	17/21	17/21
RCW[6]	1.3 ± 0.3[a]	2.0 ± 0.3[a,b]	1.6 ± 0.3[b]	1.9 ± 0.4
Caecal fusiforms (%)	83 ± 10[a]	71 ± 12[a,b,c]	79 ± 9[b]	82 ± 10[c]

[1-3,6] See legend to Table 7.

In experiment 2, the addition of ground, whole *Phaseolus* beans to the natural-ingredient diet again significantly raised the SFB score (Table 8), the effect being greater than in experiment 1. Both the skin and kernel fraction in the diet elevated the SFB score when compared with the diet without *Phaseolus*. When compared with whole *Phaseolus* beans, neither the skin nor kernel fraction affected the SFB score.

The addition of whole *Phaseolus* to the diet in experiment 2 significantly elevated relative caecum weight and lowered the percentage of caecal fusiforms (Table 8). The former effect was mimicked by the kernel fraction. This suggests that non-fibre carbohydrates in the kernel fraction (cf. Table 3) caused enlargement of the caecum. When compared with whole *Phaseolus*, both the skin and kernel fraction significantly raised the percentage of caecal fusiforms.

DISCUSSION

The feeding of raw *Phaseolus* beans is known to have adverse effects on the small intestine in rats, which is caused by the phytohaemagglutinin component [15-18]. In this study with mice, the diets containing raw, ground whole or fractionized *Phaseolus* beans did neither induce weight loss nor growth retardation, and did not cause diarrhoea or lesions in the small intestine. Based on the phytohaemagglutinin concentrations in the *Phaseolus* beans used [8], the challenge with phytohaemagglutinin was comparable to that in a rat study showing adverse effects [15]. Thus, it appears that mice are less sensitive to dietary phytohaemagglutinin than are rats.

In the course of the two experiments, SFB scores fell from about 50 at the beginning of the experiments to a value of 2 at the end of the experiments in mice fed the natural-ingredient diet without *Phaseolus*. This may reflect an effect of ageing [6]. Such an effect was not shown for the percentage of caecal fusiforms. This indicates that SFB scores and caecal fusiforms are not associated. Likewise, taken all dietary groups together, there was no correlation of SFB scores with either relative caecum weight or faecal Enterobacteriaceae. This is in accordance with the results of earlier studies [5, 8, 12].

As would be expected [5], the purified diet without *Phaseolus* did not allow SFB colonization. The addition of whole *Phaseolus* to the natural-ingredient diet systematically caused an increase of the SFB score and incidence of SFB-positive animals. This effect was not observed with the purified diet. Possibly, the purified diet lacks a factor which is necessary for SFB colonization, while supplementation with *Phaseolus* does not replenish this factor.

The results of experiment 2 indicate that whole *Phaseolus* beans and its skin and kernel fraction were equally effective in stimulating SFB appearance. The diets were formulated so that the diets with either skin or kernel fraction contained an amount of these fractions identical to that in the diet containing whole *Phaseolus*. Possibly, in experiment 2 a maximum degree of SFB colonization was reached, which would imply that both the skin and kernel fraction of *Phaseolus* contain a similar amount of SFB-inducing factors. In any event, *Phaseolus* beans do not contain SFB-inducing factors that are sufficiently effective to produce SFB colonization in mice fed a purified diet containing *Phaseolus*. Alternatively, one or more of the components omitted from the diet in favour of *Phaseolus*, that is skim milk powder, ground barley and wheat middlings, may contain SFB-inhibiting factors. The almost identical SFB scores in mice fed the diets with either whole *Phaseolus*, skin or kernel fraction, would imply that there was no dose-dependent inhibitory effect of the three components.

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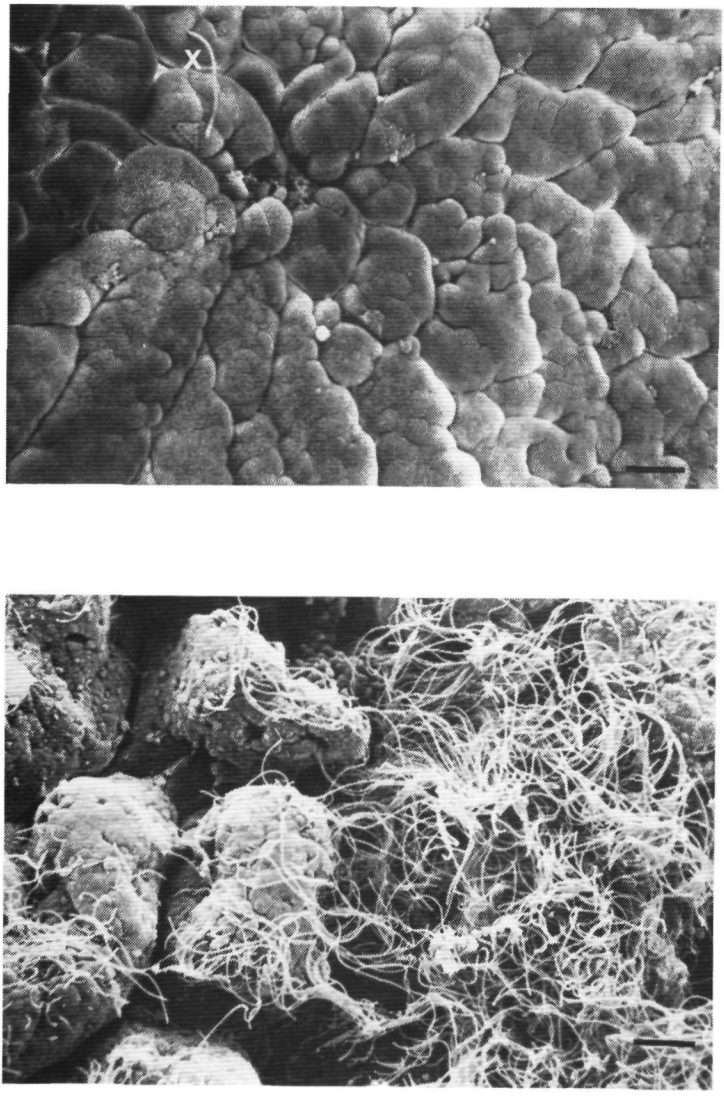

Fig. 1. Scanning electron micrographs of segmented, filamentous bacteria (SFBs) attached to the ileal mucosa of a mouse fed a natural-ingredient diet either without *Phaseolus* (A) or with *Phaseolus* (B).

(A) A single SFB (X) attached to the epithelium of a Peyer's patch. Bar = 18 μm. (B) Villi with attached SFBs (left) and a dense mat of SFBs covering the Peyer's patch epithelium (right). Bar = 31 μm.

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2.7 Influence of antimicrobial drugs on segmented filamentous bacteria in the ileum of mice

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SUMMARY

The effects of various types of 3-day antibiotic treatment of mice on the presence of segmented filamentous bacteria (SFBs) in the ileum were measured in order to further characterize these microorganisms. To assess any specific effects of the antimicrobial drugs on SFBs, relative caecal weight, relative number of fusiform-shaped bacteria in the caecum and the number of faecal Enterobacteriaceae were also determined. All drugs tested, i.e. amoxicillin, doxycyclin, gentamicin, vancomycin, ciprofloxacin, trimethoprim, metronidazol, clindamycin, streptomycin and cefotaxim, reduced the presence of SFBs in the ileum, though to different degrees. None of the drugs affected body weight of the mice. There was no correlation of the drug effects on SFBs and those on either relative caecal weight, percentage of caecal fusiforms or faecal Enterobacteriaceae. Thus, the effects of the antimicrobial drugs on SFBs can be considered rather specific. The sensitivity pattern of SFBs suggests that they are facultatively anaerobic bacteria with relatively high sensitivity to antimicrobial drugs.

INTRODUCTION

Because they cannot be cultured *in vitro*, segmented filamentous bacteria (SFBs), inhabiting the distal small intestine of mice are poorly characterized.[1,3,11] These bacteria have also been demonstrated in animal species other than mice.[5,6,9] SFBs might influence the host's resistance to certain enteropathogenic bacteria.[5,9,19,24,27] Habitat and morphology of SFBs have been studied[1,3,4], but metabolic characteristics are unknown.

SFBs in mice and rats are resistant to some antimicrobial drugs (AD) but sensitive to others.[4,8,15,16,18,20] The influence of AD on SFBs has not been investigated systematically. Therefore, we determined a sensitivity pattern of SFBs towards ten different AD. It was anticipated that the information thus obtained would contribute to the functional characterization of SFBs. In order to assess the specificity of the response of SFBs to treatment of mice with AD, we also measured three general parameters of the intestinal microbial ecology[18,20] : relative

caecal weight (RCW), percentage of fusiform-shaped bacteria in the caecum and number of faecal Enterobacteriaceae.

MATERIALS AND METHODS

Antimicrobial drugs

We selected 10 AD, representing nine categories of AD. The following AD were used. Aminopenicillins: Na-amoxicillin (Clamoxyl^{R}; Beecham Farma BV, Amstelveen, The Netherlands); tetracyclins: doxycyclin hydrochloride (Vibramycin^{R} I.V.; Pfizer BV, Rotterdam, The Netherlands); aminoglycosides: gentamicin sulphate (Garamycin^{R}; Essex Laboratories BV, Heist-op-den-Berg, Belgium) and streptomycin sulphate (Streptomycin sulphate^{R}; Pharmachemie BV, Haarlem, The Netherlands); polypeptide antibiotics: vancomycin hydrochloride (Vancocin^{R}; Eli Lilly Int Corp, Indianapolis, USA); quinolones: ciprofloxacin lactate (Ciproxin^{R}; Bayer, Leverkusen, FRG); diaminopyrimidines: trimethoprim (Trimethoprim^{R}; Pharmachemie BV, Haarlem, The Netherlands); nitroimidazol derivatives: metronidazol (Metronidazol^{R}; NPBI, Emmer-Compascuum, The Netherlands); lincomycin group: clindamycin phosphate (Dalacin C^{R}; Upjohn, Ede, The Netherlands) and cephalosporins: Na- cefotaxim (Claforan^{R}; Roussel BV, Hoevelaken, The Netherlands).

Table 1 shows the dosage of AD given to mice in three separate experiments. The doses in experiment 1 were based on human therapeutic dosage, except for streptomycin which was dosed according to other investigators.[7,8,18,25,26] Cefotaxim was injected intraperitoneally. All other AD were dissolved in drinking water in amounts equivalent to about one human daily dose per liter. The concentrations in experiments 2 and 3 were 10% and 1% of those in experiment 1 (Table 1).

To improve taste, *sirupus rubi idaei* (Brocacef BV, Maarssen, The Netherlands) was added to the drinking water to concentrations of either 25 or 40% (v:v). The former concentration corresponded to ca. 160 g saccharose/l water. To take into account any effects of the syrup, one control group received drinking water with 25% syrup (Table 1). The AD were administered during 3 d.

Table 1 Dosage of antimicrobial drugs administered to mice

Antimicrobial drug	Mg/ml drinking water or mg/mouse/day*		
	Expt 1	Expt 2	Expt 3
Amoxycillin†	3 0 (18 40)	0 3 (2 50)	0 03 (0 29)
Doxycyclin†	0 22 (2 20)	0 022 (0 16)	0 002 (0 02)
Gentamicin†	0 3 (2 50)	0 03 (0 25)	0 003 (0 03)
Vancomycin†	0 75 (4 20)	0 075 (0 25)	0 0075 (0 06)
Ciprofloxacin†	0 75 (5 40)	0 075 (0 62)	0 0075 (0 08)
Trimethoprim†	0 38 (3 80)	0 038 (0 34)	0 0038 (0 04)
Metronidazole‡	1 5 (4 10)	0 15 (0 32)	0 015 (0 10)
Clindamycin†	0 08 (0 80)	0 008 (0 04)	0 0008 (0 009)
Streptomycin†	5 0 (28 00)	0 5 (4 40)	NS
Cefotaxim†§	— (10 00)	— (1 00)	— (0 10)
Control‖	—	—	—
Control with syrup¶	NS	—	—

*Dose expressed as mg mouse day is given in parentheses
†Drinking water also contained 25 per cent (v v) syrup
‡Drinking water also contained 40 per cent (v v) syrup
§Cefotaxim was administered intraperitoneally
‖Drinking water without additives
¶Drinking water with 25 per cent (v v) syrup
NS = not studied

Animals and housing conditions

The number of mice used is indicated in Table 2. Female Cpb:SE (Swiss) mice, 7-wk old and with an SPF flora[22] , were used. Body weight was on average 20 g. The mice were housed individually in wire-topped type II macrolon cages (RUCO Metaalindustrie Nederland BV, Valkenswaard, The Netherlands). All mice were supplied *ad libitum* with demineralized sterilized water and a home-made[17] pelleted diet. Room temperature was 20-22°C, relative humidity 60-70% and light was on from 06.00 - 18.00 h.

Measurements

Individual body weights were measured daily. Daily water consumption per animal was measured to determine the intake of AD. After 3 d, all mice were killed by cervical dislocation. The presence of SFBs in the ileum (SFB score) was assessed by light microscopic examination of Gram-stained mucosal scrapings[11], and was expressed as the percentage of SFB-positive fields; 100 fields were examined at a

magnification of 1000x. Caeca with contents were weighed and results (RCW) expressed as percentage of body weight. With Gram-stained microscopic slides (magnification 1000x) of caecal contents the number of fusiforms per 100 bacteria (percentage caecal fusiforms) was determined. On days -1, 1 and 3, the number of faecal Enterobacteriaceae was determined for individual mice.[14]

Statistical analysis

Differences between group means within each experiment were statistically evaluated using Kruskal-Wallis test. When groups differed significantly, the Wilcoxon test was applied to compare test and control groups. SFB scores within test groups were also compared between experiments. Within each experiment, the number of faecal Enterobacteriaceae for the different days were compared between groups. Kendall correlation coefficients were calculated between SFB scores and the other three parameters. $P<0.05$ was defined as statistically significant.

RESULTS

Treatment with AD did not affect body weights (data not shown). Table 1 describes the calculated intakes of AD. Daily water consumption per treated mouse ranged between 3 and 10 ml. Water consumption of the control mice given drinking water without additives was 6-8 ml. The control mice with drinking water containing 25% (v:v) syrup drank 10-20 ml/d. Thus, the syrup increased water consumption.

In the treated mice of experiment 1, SFBs were not detectable (Table 2). SFB-harbouring mice were present only in the control group (Table 2). In experiment 2, using ten-fold lower AD concentrations, SFBs were still absent in treated mice except for those treated with either trimethoprim or metronidazol (Table 2). Because of the small numbers of mice per group the inhibitory effect of AD on SFB appearance seen in experiments 1 and 2 could not be substantiated statistically. In experiment 3, all AD, except for gentamicin, trimethoprim and metronidazol, produced significantly reduced SFB scores compared with those of the two control groups ($P<0.0001$). In experiment 3, SFB scores of the two control groups differed significantly ($P=0.004$). SFB scores in mice treated with gentamicin

(P=0.020) were significantly higher in experiment 3 than in experiments 1 and 2. In experiments 2 and 3, mice given either trimethoprim or metronidazol had significantly higher SFB scores than in experiment 1.

Irrespective of the dose studied, amoxicillin, doxycyclin, ciprofloxacin, clindamycin, streptomycin and cefotaxim induced absence of SFBs in mucosal smears of all mice treated (Table 2). The addition of syrup to the drinking water did not influence the incidence of SFB-positive mice.

Table 2 Influence of antimicrobial drugs on SFBs in mice

Antimicrobial drug†	Expt 1		Expt 2		Expt 3	
	SFB score‡	Incidence§	SFB score‡	Incidence§	SFB score‡	Incidence§
Amoxycillin	ND	0/2	ND	0/2	ND* **	0/8
Doxycyclin	ND	0/2	ND	0/2	ND* **	0/8
Gentamicin	ND*	0/2	ND*	0/2	35±22[b]	13/14
Vancomycin	ND	0/2	ND	0/2	0·3±0·5* **	4/14
Ciprofloxacin	ND	0/2	ND	0/2	ND* **	0/8
Trimethoprim	ND*	0/2	37±27[b]	8/8	12±21* *	6/8
Metronidazole	ND*	0/2	10±14[b]	7/8	33±23[b]	7/8
Clindamycin	ND	0/2	ND	0/2	ND* **	0/8
Streptomycin	ND*	0/6	ND*	0/6	NS	
Cefotaxim	ND	0/2	ND	0/2	ND* **	0/8
Control	18±4	2/2	42±32	6/8	44±20	8/8
Control with syrup	NS		15±21	1/2	17±18*	7/8

†For dosage of antimicrobial drugs see Table 1
‡Percentage SFB-positive fields in Gram-stained mucosal smears of the ileum means ± SD
§Number of SFB positive mice total number of mice
ND = not detectable NS = not studied
Kruskal Wallis test, P values 0·005 (expt 1), 0·0012 (expt 2) <0·0001 (expt 3), *significantly different from mice in control group of the same experiment **significantly different from mice in group control with syrup of the same experiment, Wilcoxon test, $P<0·05$
Values in the same row with different superscript letters (a b) are significantly different (Wilcoxon test, $P<0·05$)

Table 3 shows that RCW was not systematically affected by the AD. Doxycyclin and trimethoprim did not alter percentage caecal fusiforms ($P<0.05$). Lowering the dosage of the other AD was associated with increased percentage fusiforms. At the lowest dosage (Expt 3), the AD did not decrease percentage caecal fusiforms, except for amoxicillin.

Table 4 illustrates that AD treatment differently influenced the number of faecal Enterobacteriaceae. At the lowest dosage (Expt 3) only ciprofloxacin systematically reduced this parameter, whereas at the highest dosage gentamicin, streptomycin and cefotaxim also did so.

Table 3. Influence of antimicrobial drugs on relative caecal weight and percentage fusiform-shaped bacteria in the caecum of mice

Antimicrobial drug†	Relative caecal weight‡			Percentage fusiforms§		
	Expt 1	Expt 2	Expt 3	Expt 1	Expt 2	Expt 3
Amoxycillin	5·4 ± 0·2	2·8 ± 0·0	2·1 ± 0·7	ND	13 ± 11*	33 ± 26*
Doxycyclin	2·7 ± 0·5	2·3 ± 0·0	1·9 ± 0·6	82 ± 23	73 ± 33	60 ± 19
Gentamicin	3·2 ± 0·6	2·5 ± 0·2	2·0 ± 0·5	ND	10 ± 7*	70 ± 12**
Vancomycin	4·9 ± 0·1	3·0 ± 0·0	2·3 ± 0·4	ND	5 ± 0*	52 ± 26
Ciprofloxacin	3·6 ± 1·6	2·6 ± 0·7	1·6 ± 0·5	ND	83 ± 11	81 ± 10**
Trimethoprim	1·5 ± 0·1	1·9 ± 0·4**	2·2 ± 0·6	85 ± 7	75 ± 12*	64 ± 12
Metronidazole	4·5 ± 1·5	2·2 ± 0·4**	1·9 ± 0·3	23 ± 25	64 ± 31	70 ± 12
Clindamycin	3·0 ± 0·7	3·1 ± 0·5	2·0 ± 0·6	25 ± 35	25 ± 35*	83 ± 13*·**
Streptomycin	3·2 ± 0·7	2·8 ± 0·6	NS	21 ± 22	40 ± 22	NS
Cefotaxim	4·5 ± 1·6	2·1 ± 0·4	2·0 ± 0·6	33 ± 46	73 ± 33	75 ± 12**
Control	2·9 ± 0·0	2·1 ± 0·7**	2·1 ± 0·6	95 ± 0	84 ± 12**	68 ± 18
Control with syrup	NS	1·0 ± 0·0	2·0 ± 0·0	NS	50 ± 0	57 ± 9

†See legend to Table 2.
‡Caecum weight expressed as percentage of body weight; means ± SD.
§Number of fusiform-shaped bacteria per 100 bacteria; means ± SD.
ND = not detectable; NS = not studied.
Kruskal–Wallis test, *P* values: relative caecal weight, 0·11 (expt 1), 0·010 (expt 2), 0·20 (expt 3); percentage fusiforms, 0·020 (expt 1), 0·005 (expt 2), 0·0001 (expt 3); *,**see legend to Table 2.

Table 4. Influence of antimicrobial drugs on faecal Enterobacteriaceae in mice

Antimicrobial drug†	Log_{10} (number of Enterobacteriaceae/g faeces)‡								
	Expt 1			Expt 2			Expt 3		
	Day − 1	Day 1	Day 3	Day − 1	Day 1	Day 3	Day − 1	Day 1	Day 3
Amoxycillin	3·0	3·0	1·5	ND	5·5	8·0*	2·5	2·0*	3·3
Doxycyclin	5·0	3·5	2·0	ND	3·0	4·5	4·2	4·0	5·0
Gentamicin	4·0	ND	ND	4·0	3·5	1·5	3·7	5·0	3·0
Vancomycin	4·0	6·5	6·0	3·5	3·0	7·0*	3·5	3·7	3·2
Ciprofloxacin	4·5	ND	ND	2·0	ND	5·0	2·8	ND*	ND*
Trimethoprim	5·0	5·0	2·0	3·8	3·4	3·8	NA	NA	NA
Metronidazole	5·0	3·5	6·0	3·9	4·1	4·3	NA	NA	NA
Clindamycin	4·5	4·5	5·6	3·0	8·0	7·0	3·3	4·3	4·8
Streptomycin	3·2	ND*	ND*	2·0	ND*·**	ND*·**	NS	NS	NS
Cefotaxim	2·5	ND	ND	1·5	4·5	3·5	3·8	2·7*	3·2
Control	4·5	4·5	2·5	4·0	3·6	3·5	5·0	4·7	4·2
Control with syrup	NS	NS	NS	3·0	3·5	4·5	NA	NA	NA

†See legend to Table 2.
‡Group means.
NA = not analysed; ND = not detected; NS = not studied.
Kruskal–Wallis test, *P* values < 0·025; *,**see legend to Table 2.

DISCUSSION

This study clearly shows that SFB scores, RCW, caecal fusiforms and faecal Enterobacteriaceae respond differently to AD. This is supported by the fact that SFB scores and none of the three parameters were significantly correlated. Differential responsiveness to external factors for SFB scores and either RCW or caecal fusiforms was reported earlier.[12,13] As to a correlation between SFBs and Enterobacteriaceae there are conflicting studies.[12,13,21] Thus, the AD studied may have direct effects on SFB scores either by inhibiting SFB colonization or by killing SFBs.

SFBs were sensitive to amoxicillin, doxycyclin, vancomycin, ciprofloxacin, clindamycin and cefotaxim, even given in lower doses than used normally by other investigators.[8,29] Streptomycin given at therapeutic concentrations[7,8,18,25,26,29] or 10x lower also eliminated SFBs. The high sensitivity of SFBs to some of the AD tested agrees with earlier findings.[4,18,20,28] It has been reported that roxythromycin, erythromycin, neomycin, streptomycin, bacitracin, the combination trimethoprim-sulfamethoxazole, and polymyxin induce disappearance of SFBs from the ileum of mice.[18,20] Within 10.5 h of access to drinking water containing 0.6 mg/ml penicillin, mice were free from SFBs.[4] SFBs were scarcely present in the ileum of broiler chickens treated with virginiamycin.[28]

Table 5 Simplified pattern of sensitivity to antimicrobial drugs of SFBs, compared to that of facultatively anaerobic and obligately anaerobic bacteria

Antimicrobial drug	Sensitivity of				Degree of sensitivity of SFBs†
	Facultative anaerobes*		Obligate anaerobes*		
	Gram +ve	Gram −ve	Gram +ve	Gram −ve	
Amoxycillin	+	v	+	−	+++
Doxycyclin	+	+	+	−	+++
Gentamicin	v	+	−	−	++
Vancomycin	+	−	+	−	++
Ciprofloxacin	+	+	−	−	+++
Trimethoprim	+	v	−	−	+
Metronidazole	−	−	+	+	+
Clindamycin	v	v	+	+	+++
Streptomycin	+	v	−	−	++‡
Cefotaxim	+	+	+	−	+++

*+, Sensitive, −, insensitive, v, variation in sensitivity between bacterial species (source McEvoy, GK (ed) AHFS Drug Information 90, American Society of Hospital Pharmacists, 1990)
†+ Elimination of SFBs by dose ≥therapeutic dose (TD), ++, elimination by dose ≥TD × 10^{-1}, +++, elimination by dose ≥TD × 10^{-2}
‡Sensitivity of SFBs to streptomycin given at a dose TD × 10^{-2} not tested

Table 5 summarizes the observed *in vivo* sensitivity of SFBs to AD, compared to the *in vitro* sensitivity of facultative and obligate anaerobes. SFBs were inhibited by doses of ciprofloxacin as low as 1% of the therapeutic dose, whereas obligately anaerobic bacteria are insensitive.[23] This also holds for gentamicin[2,23] and streptomycin[2,23] whereas these drugs, given at 10% of the therapeutic mouse dose, inhibited SFBs. Thus, SFBs behave unlike obligate anaerobes.

Based on the sensitivity pattern and the fact that the habitat of SFBs is the relatively anaerobic ileum,[3] they can be considered facultatively anaerobic with high sensitivity to AD.

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Chapter 3

Isolation of SFBs

3.1 Colonization of germ-free mice by segmented filamentous bacteria after oral administration of various murine intestinal wall preparations

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SUMMARY

Intestinal suspensions containing segmented filamentous bacteria (SFBs) were given orally to germ-free mice in an attempt to pave the way for the production of mice mono-associated with SFBs. The effects of dilution, sonication and chloroform/ethanol treatment of intestinal suspensions on SFB colonization were investigated. Colonization density of SFBs in the germ-free mice was dependent on the number of SFBs in the administered suspension. Sonication of suspensions had no effect. Chloroform/ethanol treatment of suspensions resulted in the production of di-associated mice, containing both SFBs and a non-characterized *Clostridium* in their small intestine. It is suggested that, through specific elimination of the *Clostridium*, these di-associated animals may be used to produce mice containing SFBs alone.

INTRODUCTION

Segmented filamentous bacteria (SFBs) that are attached to the ileal mucosa, may play a role in the development of immunity to gastrointestinal infections in mice, rats, domestic fowls and dogs.[2-4] SFBs have only been characterized morphologically, because so far they cannot be cultured *in vitro*.[5,7,13-15] Colonization of the small intestine by SFBs depends on intimate adherence to the mucosa.[1,4,14] Possibly, SFBs can only be cultured *in vivo*, i.e. as monocultures in animals.

The present preliminary studies were carried out in order to obtain clues as to possible ways to produce mice that are relatively rich in SFBs and poor in other microorganisms. For this purpose germ-free animals were inoculated with various suspensions containing SFBs. Murine intestinal wall suspensions were treated differently with the objective to increase the relative proportion of SFBs. Suspensions were sonicated to divide ('disrupt') SFB chains, which consist of up to 90 segments[1,14] , thereby increasing the number of viable SFB units per ml suspension. Since SFBs are resistant to chloroform and ethanol[10] , murine intestinal wall suspensions were also treated with these organic solvents in order to enrich the suspensions with SFBs.

MATERIALS AND METHODS

Animals

As donor mice for intestinal material we used 8wk-old female Cpb:SE (Swiss) mice, of SPF quality. Microbiological quality of the mice and their housing conditions have been described.[12] The mice to be inoculated were 6 to 8wk-old germ-free Cpb:SE mice. The mice given sonicated suspensions were housed individually for 7d on sawdust in macrolon cages Type S (RUCO Metaalindustrie Nederland BV, Valkenswaard, The Netherlands), provided with a filter cap. A natural ingredient, pelleted diet[11] and water (acidified, pH 2.3) were supplied *ad libitum*. Room temperature was 20-22°C and relative humidity 60-70%. Light was provided from 06.00-18.00 h. The mice given chemically treated suspensions were kept under germ-free conditions in a Trexler-type isolator and supplied *ad libitum* with an autoclaved, pelleted diet[11] and demineralised, sterilised water. Environmental conditions were as described for the other animals.

SFB-containing suspensions

Five donor mice were killed by cervical dislocation, the small intestines removed and divided into nine equal sections as described.[9] Sections 7 and 8 were removed and flushed with 1.0 ml of a sterile 0.9% NaCl solution, and homogenized in broth[8] with an Ultraturrax^{R} homogeniser (TP 18.10; Janke and Kunkel, Staufen im Breisgau, FRG). The number of SFBs per ml suspension was determined microscopically in 0.01 ml which was spread out on 1 cm^2 of a microscopic slide and stained with methylene blue. From the mean number of SFBs per field (20 fields, magnification 1000x) the number of SFBs per ml was calculated.

Part of the suspension was used to prepare two identical dilution series (dilution factors: 10^{-1}, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}; dilution fluid: broth[8]). One series was treated with a Kontes^{R} 881430 ultrasonic cell disruption system (Scientific Glassware/Instruments, Vineland, N.J. USA), during 30s with tip amplitude 24 μm. The other series served as untreated control.

Another part of the suspension was treated successively as follows: chloroform

3% (incubation time, 1h), ethanol 80% (4h), and diluted in tenfold steps (10^{-1} - 10^{-8}). These dilutions were incubated and subcultured on enriched blood agar plates[6] at 37°C in an anaerobic glove box (ITL International BV, Leek, The Netherlands) as described.[6] All procedures were performed aseptically.

Inoculation

From each dilution of the sonicated and untreated series 1.0 ml was administered orally to one germ-free male and one female mouse.

One dilution of the chemically treated series was selected for administration. The suspension with the lowest dilution factor, but containing maximally one non-SFB species, was administered orally to eight germ-free mice of both sexes (0.5 ml per mouse). The number of SFBs per ml suspension was determined as described above.

Intestinal parameters of inoculated mice

After 7d the twenty mice given diluted suspensions either sonicated or untreated, were killed by cervical dislocation and weighed. Colonization of SFBs was determined by preparing Gram-stained slides of mucosal scrapings of intestinal sections 5-9. Colonization density (SFB score) was expressed as the integrated number of SFB-positive fields per 100 fields (for each mouse five sections and twenty fields per section were examined; magnification 1000x). In each mouse the mean percentage of SFBs (number of SFBs in a sample of 100 bacteria) was determined in slides of intestinal sections 8 and 9 (magnification 1000x). From each mouse the caecum with contents was removed and weighed. Relative caecal weight was calculated as percentage of body weight. A Gram-stained slide of caecal contents was prepared and the percentage of fusiform-shaped bacteria determined.

Two mice inoculated with the chloroform/ethanol treated suspension were killed days 8, 40 and 150, respectively. SFB scores and percentage intestinal SFBs were determined as described above. The identity of other intestinal bacteria was established by means of Gram-stained mucosal slides of the small bowel and by culturing contents from intestinal sections 8 and 9 and the caecum.

RESULTS AND DISCUSSION

The colonization density of SFBs in germ-free mice inoculated with intestinal wall suspensions was dose-dependent; a dose of 10^4 or less resulted in very low SFB scores (Table 1). The percentage of SFBs was influenced similarly (Table 1). This dose-effect relationship is unlike normal behaviour of cultured bacteria which multiply exponentially within 24-48h. Possibly, SFBs react differently from other microorganisms due to their dependency on the epithelial cells of the small intestinal mucosa.

Sonication of the administered suspensions did not influence the colonization pattern (Table 1). Besides, sonication of the diluted suspensions did not result in elimination of unwanted bacteria. It can be concluded that the combination of sonication and dilution is unsuitable to increase the relative concentration of SFBs in these suspensions.

Table 1 Ileal SFB scores and percentages of SFBs in germ-free mice 7 d after oral administration of diluted intestinal wall suspensions that were either untreated or sonicated

	SFB score*		Percentage SFBs†	
Dose‡	Control suspensions	Sonicated suspensions	Control suspensions	Sonicated suspensions
10^7	82, 94	67, 42	75, 78	74, 24
10^6	86, 0	69, 73	73, 0	45, 53
10^4	6, 0	3, 0	1, 0	1, 0
10^2	2, 0	3, 0	0, 0	0, 0
10^0	0, 0	0, 0	0, 0	0, 0

*Number of SFB positive fields per 100 fields (determined in small intestinal sections 5-9, magnification 1000 ×)
†Number of SFBs counted in a sample of 100 bacteria at the small intestinal mucosa, for each mouse is given the mean value of the numbers determined in small intestinal sections 8 and 9
‡Number of SFBs administered per mouse
Individual values are given, two mice were inoculated with either control or sonicated suspensions

Clear influences of dilution and sonication of the intestinal wall suspension on relative caecal weight and percentage caecal fusiforms were not observed. Relative caecal weight tended to be lower in mice inoculated with the highest dose of bacteria (Table 2). As to the relative presence of fusiform-shaped bacteria in caecum there was an opposite tendency for control suspensions.

Table 2 Relative caecal weight and percentage of caecal fusiform-shaped bacteria 7 d after oral administration of diluted intestinal wall suspensions that were either untreated or sonicated

Dose†	Relative caecal weight		Percentage caecal fusiforms*	
	Control suspensions	Sonicated suspensions	Control suspensions	Sonicated suspensions
10^7	2 3, 2 2	1 8, 2 7	96, 96	74, 80
10^6	2 6, 4 1	2 2, 3 5	66, 40	40, 20
10^4	2 6, 3 4	5 2, 4 6	50, 20	50, 52
10^2	4 2, 3 1	2 4, 4 9	97, 50	96, 68
10^0	3 1, 2 9	3 8, 4 8	74, 80	52, 100

*Number of fusiform-shaped bacteria in a sample of 100 caecal bacteria
†Number of SFBs administered per mouse
Individual values are given, two mice were inoculated with either control or sonicated suspensions

As shown earlier[10] , SFBs survived treatment with chloroform and ethanol. However, spore-forming bacteria are also insensitive to the treatment. In mice examined 8, 40 and 150d after inoculation, about half of the microorganisms found in intestinal section 7, were SFBs. In the other sections the SFBs were outnumbered by anaerobic Gram-positive spore-forming and rod-shaped bacteria, i.e. bacteria belonging to a *Clostridium* species (data not shown).

This study has shown that sonication of intestinal wall suspensions and subsequent administration to germ-free mice is unsuitable to produce mice mono-associated with SFBs. However, by chloroform/ethanol treatment of the suspensions di-associated mice, possessing SFBs and a *Clostridium*, can be obtained. Possibly, this *Clostridium* can be eliminated by specific antibiotic treatment, micro-manipulation, using anti-*Clostridium* antibodies or by other methods.

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3.2 Mono-association of mice with non-cultivable, intestinal, segmented, filamentous bacteria

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SUMMARY

A technique is described so that mice mono-associated with non-cultivable, segmented filamentous bacteria (SFBs) can be produced for the first time. As SFB donors, mice were used which had an intestinal microflora consisting of both SFBs and bacteria of the genus *Clostridium*. Recipients were germ-free mice. It was demonstrated that the intraileal inoculation method was more effective than the orogastric route. Therefore, intestinal homogenates of donor mice were treated with filtered ethanol, diluted and administered intraileally to recipient mice. Evidence is presented that cage mates of the recipient mice were mono-associated with SFBs. The availability of these animals, i.e. *in vivo* monocultures of SFBs, allows taxonomic and functional characterization of SFBs, which was as yet not possible.

INTRODUCTION

Autochthonous, segmented, filamentous bacteria (SFBs) inhabiting the ileum of mice and rats have been extensively studied morphologically by scanning and transmission electron microscopy (Davis and Savage 1974, Savage and Blumershine 1974, Chase and Erlandsen 1976, Blumershine and Savage 1978, Snellen and Savage 1978, Ferguson and Birch-Andersen 1979, Koopman *et al.* 1987). Functional characterization and identification of the bacterium have been hampered because attempts to culture SFBs *in vitro* were unsuccessful (Savage and Blumershine 1974, Koopman *et al.* 1984, 1988, Merrell *et al.* 1979, Tannock *et al.* 1987). In addition, the possible significance of SFBs to the host remains unclear. There are some indications that SFBs may play a role in the gastrointestinal colonization resistance against pathogenic invaders (Merrell *et al.* 1979, Garland *et al.* 1982, Koopman *et al.* 1984).

In a preliminary attempt to produce mice mono-associated with SFBs we prepared intestinal homogenates derived from specified pathogen-free (SPF) mice colonized with SFBs, treated these homogenates with ethanol and chloroform and administered dilutions of treated homogenates to germ-free mice (Klaasen *et al.* 1990b). This procedure resulted in mice associated with both SFBs and clostridia.

With the use of these mice as SFB donors, we now describe a technique to produce mice mono-associated with SFBs.

MATERIALS AND METHODS

Animals and housing

Germ-free, Cpb:SE (Swiss) mice of both sexes, aged 4 weeks, and kept in a Trexler type plastic isolator under conditions as described (Klaasen *et al.* 1990b), were used throughout as SFB recipients. Donor mice used to determine the minimum dose of SFBs necessary to colonize the ileum of recipient mice (experiment 1) were female Cpb:SE mice, aged 5 weeks, with an SPF flora. Microbiological quality of these mice and their housing conditions have been described elsewhere (Koopman *et al.* 1989b). SFB donor mice used to convert germ-free mice into mice mono-associated with SFBs (experiment 2) were 5 week-old Cpb:SE mice of both sexes with an intestinal microflora consisting of SFBs and some bacterial species from the genus *Clostridium*. These donor mice were produced and housed as described (Klaasen *et al.* 1990b).

After inoculation of the recipient mice in experiment 1, they were housed individually on sterilized commercial sawdust (Woody-Clean type 3/4^{R}, J. Rettenmaier und Söhne, Ellwangen-Holzmühle, Germany) in type S macrolon cages (RUCO Metaalindustrie Nederland BV, Valkenswaard, The Netherlands), provided with a filter cap. In experiment 2, the inoculated mice were placed back into the isolator and housed together with untreated, germ-free counterparts in type III macrolon cages. Environmental conditions in the isolator were as described earlier (Klaasen *et al.* 1990b).

All mice received a sterilized, pelleted diet (Koopman *et al.* 1989a) and sterilized, demineralized water *ad libitum*.

Experiment 1: Determination of minimum 'infectious' dose of SFBs and effective route of inoculation

Preparation of intestinal homogenates

Eight SPF donor mice were killed by cervical dislocation. From each mouse, part of the small intestine, with a length of 12 cm and the lower end being located at 1 cm from the caecum, was removed. The intestinal contents were gently removed with a pair of tweezers and the pooled intestines (0.7 g, wet weight) homogenized in 4.5 ml of broth (Koopman *et al.* 1983) with the use of an Ultraturrax^R homogenizer (TP 18.10; Janke and Kunkel, Staufen im Breisgau, FRG). This homogenate was stepwise diluted by a factor 10 up to 6 times using the broth as diluent. The concentration of SFBs in the dilutions was determined as follows. 0.01 ml of dilution was spread on 1 cm^2 of a microscopic slide and Gram-stained. With a light microscope, the number of SFBs was counted in 20 fields at 4 randomly chosen spots of the slide at a magnification of 1000x. With the mean number of SFBs per field, the number of SFBs per ml of each dilution was calculated.

Inoculation

SFBs were administered to germ-free mice either orogastrically or intraileally. Orogastric inoculation was performed with a blunt needle. Two different SFB doses (Table 1) were given, the inoculum volume being 0.5 ml/mouse. Intraileal inoculation was carried out as described below in experiment 2. 0.1 ml of broth containing one of three different doses (Table 1) was injected into the lumen of the ileum, at 0.5 cm from the caecum.

Examination of inoculated mice

Seven days after inoculation the recipient mice were killed by cervical dislocation. The small intestinal mucosa was examined for the level of colonization of mucosa-associated SFBs. Mucosal smears on microscopic slides were prepared as described by Klaasen *et al.* (1990a). With a light microscope, the colonization density of SFBs was determined in smears derived from five different locations (Klaasen *et al.* 1990a) in the distal half of the small bowel, and expressed as SFB score. This score is the mean percentage of SFB-positive fields out of 20 fields per smear examined at a magnification of 1000x.

Preparation and treatment of intestinal homogenates

Ten donor mice with a microflora of SFBs and clostridia were killed by cervical dislocation while in the isolator. They were then transferred, strictly aseptically, into a sterile laminar flow cabinet to collect the small intestines. From pooled small intestine sections (0.8 g, wet weight) one homogenate was prepared by adding 4.5 ml of broth as described above under experiment 1, except that it was done under strictly aseptic conditions. From the mucosal regions located proximally and distally adjacent to the intestinal section removed, mucosal smears were prepared and the SFB scores determined as described (Klaasen *et al.* 1990a). The concentration of SFBs in the homogenate was determined as described above under experiment 1. The homogenate was treated as follows. Ethanol (96%), which had been passed through a millipore filter (FP030/3^{R}, Schleicher and Schuell, Dassel, FRG) to eliminate any spore-forming bacteria, was added to the homogenate (ethanol:homogenate = 19:1, v/v) to a final concentration of 91%. This mixture was incubated for 4 h at 20°C while stirring with a Morat^{R} magnetic stirrer (Franz Morat KG, Framo^{R} - Gerätetechnik, Hochschwarzwald, FRG). Then, the ethanol was separated by centrifugation at 600 x g during 20 min. The ethanol supernatant was carefully removed and the pellet resuspended in 4.5 ml of broth (Koopman *et al.* 1983). Two portions (0.5 ml each) of this suspension were diluted with the broth in tenfold dilution steps up to 7 times. The concentration of SFBs in the undiluted suspension was determined as described above. Except for centrifugation, all procedures were carried out in a sterile laminar flow cabinet.

Selection of proper dilution for inoculation

Samples (0.1 ml) of the ethanol-treated, undiluted homogenate and of one complete dilution series were added in duplicate onto pre-reduced, enriched blood agar plates (Koopman *et al.* 1973). The plates as well as the homogenate and the dilutions were then incubated for 18 h at 37°C in an anaerobic glove box (ITL International BV, Leek, The Netherlands) as described by Koopman *et al.* (1973). Samples (0.1 ml) of the other dilution series were added in duplicate onto non pre-reduced, but

otherwise identical, agar plates. These dilutions and plates were incubated aerobically for 18 h at 37°C. After incubation all samples were examined for bacterial growth, followed by repetition of the subculturing procedure. After another 24 h all samples were examined again. To check the influence of incubation on SFB concentration, this concentration was determined in the ethanol-treated, undiluted homogenate before and after anaerobic incubation at 37°C for 42 h. The lowest dilution but one, without visible bacterial growth after either anaerobic or aerobic incubation and with sufficiently high concentration of SFBs for an effective intraileal administration was selected for inoculation of germ-free mice. This dilution was taken from the anaerobically incubated series. After concentrating this dilution which was the 10^4-fold diluted homogenate, by centrifugation and resuspending the pellet in 1.0 ml of broth, the concentration of SFBs was determined as described above.

Intraileal inoculation

Two germ-free mice were transferred under aseptic conditions from an isolator into a sterile laminar flow cabinet. They were anaesthesized as follows. 0.1 ml of 10-fold with saline diluted Hypnorm^R (Janssen Pharmaceutica, Tilburg, The Netherlands), containing 10 μg of fluanisone and 0.315 μg of phentanyl citrate, and 0.1 ml of 10-fold with saline diluted Valium 10 Roche^R (Hoffmann-La Roche BV, Mijdrecht, The Netherlands), containing 50 μg of diazepam, were separately injected intramuscularly. After general anaesthesia had been achieved, the abdomen was opened and at 0.5 cm from the ileocaecal junction an inoculum of 0.1 ml was injected into the ileal lumen. Then, the abdomen was closed by a suture of the muscle layer with silk (USP 3-0); the skin was closed with two suture clips. The two mice were transferred back, strictly aseptically, to the isolator. They were allowed to recover in abdominal position while hypothermia was prevented by placing a heating element (38°C) directly under the bottom of the isolator. After 5 h of recovery, each mouse was placed in a cage containing 6 germ-free mice. The germ-free status of these animals had been investigated previously by bacteriological and microscopic examination of pooled samples of faeces from the isolator. In addition, one of their cage mates had been killed to examine its intestine for the presence of (*in vitro*) cultivable or non-cultivable bacteria. Both

examinations resulted in the absence of bacteria which implied that SFBs were not present in the isolator previous to the experiment.

Examination of mice exposed to inoculated counterparts

Four mice of which two had been housed with one inoculated mouse and two with the other, were examined either 5, 10 or 15 days after inoculation. For this purpose, they were killed by cervical dislocation and their small intestines investigated to determine SFB scores as described above. Light micrographs of mucosal smears were prepared by means of a Leitz Orthoplan^{R} photo microscope. Scanning electron micrographs of the ileal wall, 1 cm from the ileocaecal junction, were made following the method described by Koopman *et al.* (1987). To confirm absence of bacteria other than SFBs in these mice, intestinal and caecal tissue and their contents and faecal samples of each mouse were cultured in broth (Koopman *et al.* 1983) for 24 h at 37°C and subsequently subcultured on enriched blood agar plates for 24 h at 37°C under either aerobic or anaerobic conditions. Light microscopic examination of caecal contents and of faecal samples of each mouse was performed to detect any non-cultivable bacteria other than SFBs. Thirty days after inoculation, bacteriological examination of pooled samples of faeces from the isolator was carried out to further exclude the presence of bacteria other than SFBs.

RESULTS

In order to induce colonization of germ-free mice by SFBs within seven days, $2x10^4$ SFBs had to be administered by the orogastric route and $2x10^2$ by intraileal injection (Table 1). Thus, intraileal injection was more effective.

In experiment 2, all donor mice were found to be SFB-positive. SFB scores of these animals were 87 $\pm$ 21 (mean $\pm$ SD, n=10) for the distal and 22 $\pm$ 20 for the proximal site of the intestinal section removed for preparation of the homogenate. The concentration of SFBs in the homogenate was $1.5x10^7$/ml which is equivalent to $8.4x10^7$/g intestinal tissue. Ethanol treatment and subsequent anaerobic incubation for 42 h did not change SFB concentration.

Table 1. Colonization of the small intestine of germ-free mice by administered segmented, filamentous bacteria (SFBs)

Number of SFBs administered[1]	Route of administration	SFB score[2] in recipients at 1 wk after inoculation	Incidence[3] of SFB-positive recipients
2.0×10^2	orogastric	0 ± 0	0/4
2.0×10^4	idem	29 ± 10	4/4
4.0×10^1	intraileal	0 ± 0	0/4
2.0×10^2	idem	38 ± 9	4/4
2.0×10^4	idem	39 ± 22	4/4

[1] Number of SFBs given per mouse as based on microscopic counts in the diluted intestinal homogenates.
[2] Percentage of SFB-positive fields as determined in mucosal smears of the distal small intestine; means ± SD for 4 mice per treatment.
[3] Number of SFB-positive mice/number of examined mice.

After 42 h of incubation of the ethanol-treated, undiluted and diluted homogenates, bacterial growth was visible in the undiluted, 10-fold and 10^2-fold diluted homogenates. There were Gram-positive, polymorphic, spore-forming bacteria as detected by light microscopy. The homogenates diluted 10^3 or more did not contain bacteria other than SFBs after either anaerobic or aerobic incubation. The 10^4-fold diluted homogenate was chosen for inoculation. The concentrated suspension prepared from this dilution contained 6.6×10^4 SFBs/ml, of which 0.1 ml was inoculated intraileally.

All cage mates of inoculated mice examined either 5, 10 or 15 days after inoculation were found to harbour SFBs. SFB scores were (mean ± SD, n=4): after 5 days, 15 ± 10; after 10 days, 69 ± 14; after 15 days, 65 ± 11. The appearance of SFBs in mono-associated mice as seen in Gram-stained mucosal smears by means of which SFB scores were determined, is demonstrated in Fig. 1. As a comparison, SFBs adhering to the ileal mucosa as seen by the scanning

electron microscope, are shown in Fig. 2. Aerobic and anaerobic culturing of intestinal and caecal contents and tissues and faecal samples did not show cultivable bacteria. By light microscopic examination of caecal contents and faeces non-cultivable bacteria other than SFBs could not be detected. The same results were achieved by cultural and microscopic examination of faeces, 30 days after inoculation.

DISCUSSION

Our method of isolating SFBs from mice with a microflora of SFBs and clostridia and administrating them to germ-free mice resulted in recipients mono-associated with SFBs. To our knowledge, mice colonized by SFBs only are now available for the first time. This may have important consequences for the progress in research on characteristics of SFBs such as their taxonomy and their impact on the host in terms of gastrointestinal physiology, colonization resistance and immunity.

The choice of the intraileal route for administration, which is very effective, enabled us to use the technique of dilution to eliminate other bacteria. Diluted homogenates free of detectable, undesirable bacteria still contain sufficient SFBs to induce colonization in germ-free mice. In a preliminary study (Klaasen *et al.* 1990b), intestinal homogenates of SPF mice were treated with both chloroform and ethanol to inactivate vegetative forms of bacteria. Administration of this homogenate to germ-free mice yielded animals associated with both SFBs and clostridia. It was unclear whether these clostridia originated from the SPF donor mice or had been present in the ethanol and/or chloroform. In any event, we now used ethanol which had been filtered. Chloroform, which damaged the filter, had to be omitted.

In order to produce mice mono-associated with SFBs, we used SFB donor mice which had a flora consisting of both SFBs and clostridia (Klaasen *et al.* 1990b), and the technique of intraileal administration. Possibly, when using the combination of treatment of intestinal homogenates with filtered ethanol, dilution and intraileal inoculation, SPF mice might be suitable as SFB donors. This would simplify the procedure to generate mice mono-associated with SFBs. Prerequisite might be that the SPF donor mice are heavily colonized by SFBs.

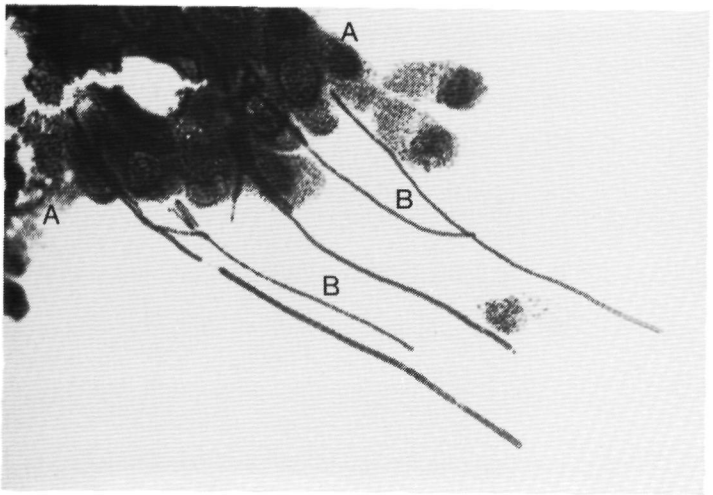

Fig.1. Segmented filamentous bacteria (SFBs) in a Gram-stained smear prepared from the ileal mucosa (1 cm from the ileocaecal junction) of a 6-week-old mono-associated mouse. **A.** Mucosal material mainly existing of epithelial cells and mucus. **B.** SFBs with one end attached to the mucosa. Light micrograph, 665x, bar = 2.4 μm.

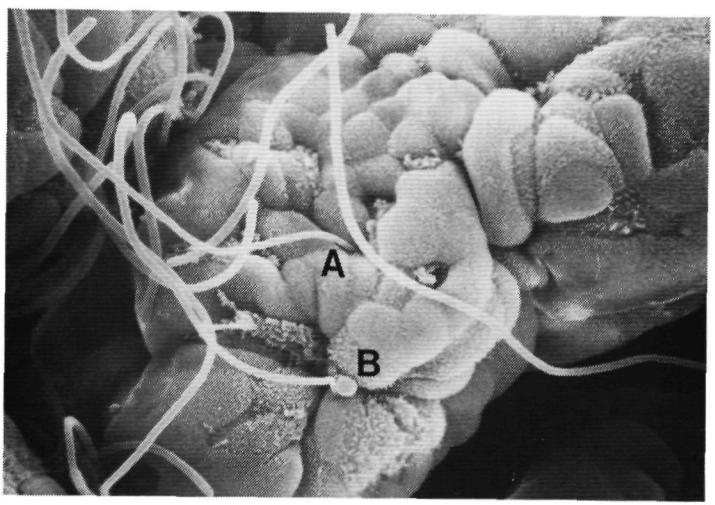

Fig. 2. Segmented filamentous bacteria (SFBs) attached to the intestinal villus epithelium (1 cm from the ileocaecal junction) of a 6-week-old mono-associated mouse. **A.** SFB with one end firmly attached to the epithelium. **B.** Lymphocyte. SEM, 1000x, bar = 3.2μm.

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Chapter 4

SFBs, the immune system and colonization resistance

4.1 Apathogenic, intestinal, segmented, filamentous bacteria stimulate the mucosal immune system of mice

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SUMMARY

Segmented, filamentous bacteria (SFBs) are apathogenic, autochthonous bacteria in the murine small intestine that preferentially attach to Peyer's patch epithelium. SFBs have never been cultured *in vitro*. We have studied the effects of SFBs on the immune system of the host. Mice mono-associated with SFBs were compared with germ-free mice, and mice associated with SFBs plus a specified pathogen-free (SPF) gut flora were compared with mice associated with the SPF gut flora only. SFBs versus no microbial flora raised the number of lymphoid cells in lamina propria of ileal and caecal mucosa, raised the number of IgA secreting cells in the intestinal mucosa, produced elevated IgA titres in serum and intestinal secretions and enhanced the concanavalin A-induced proliferative response of mesenteric lymph node cells. The SPF flora had similar effects, but less pronounced than those mediated by SFBs. The results indicate that SFBs stimulate the mucosal immune system to a larger extent than do other autochthonous gut bacteria.

INTRODUCTION

Segmented, filamentous bacteria (SFBs) are apathogenic, autochthonous bacteria that occur in the distal small bowel of various animal species (13). SFBs have never been cultured *in vitro*, and thus are not classified taxonomically (13). Ultrastructural studies have revealed details of the morphology and ecological niches of SFBs in mice and rats (2, 5, 31). Strikingly, SFBs are preferentially attached to the epithelium that covers the ileal Peyer's patches (1, 31, 32). Various authors suggested that SFBs competitively exclude pathogens from the distal small intestine, contributing to gastrointestinal colonization resistance (8, 18, 26). Others hypothesized that SFBs enhance host resistance by influencing gut-associated lymphoid tissue (9, 12). The two ideas have not yet been put to the test.

The autochthonous gut microbiota, or at least components of it, can enhance non-specific and specific immunity at the systemic and/or the mucosal level (6, 10, 27, 28, 29). For example, *Lactobacillus casei* administered per os stimulates non-specific immunity in mice by increasing the activity of peritoneal macrophages (29). Dobber *et al.* (6) suggested that intestinal bacteria are involved in the

regulation of peripheral T cells. Development of the intestinal microbiota in mice is associated with an increase in the number of duodenal IgA plasma cells, but the bacteria responsible, if any, could not be isolated and identified (28). This suggests that non-cultivable bacteria in the digestive tract of weanling mice are immunogenic. Possibly, these bacteria are SFBs.

This paper presents a study of the effects of SFBs on the immune system of their host. The study was greatly facilitated by the availability of mice mono-associated with SFBs, which we have produced recently (16).

MATERIALS AND METHODS

Experimental design

There were four experimental groups, each consisting of 15 mice. The influence of SFBs on the mucosal immune system was studied in germ-free mice and in mice with a specified pathogen-free (SPF) gut flora. Thus, we contrasted germ-free mice and mice mono-associated with SFBs and also compared SFB-free, SPF mice with SFB-positive, SPF mice. Two weeks after producing the experimental groups by selective microbial association, the microbial status was checked and various immunological parameters examined.

Animals

SPF and germ-free Cpb:SE (Swiss) mice bred in-house were used. They were aged 6-8 weeks and of both sexes. Microbiological quality and housing conditions of these mice have been described (23). Mice mono-associated with SFBs were obtained and housed as described (16). To produce mice with an SFB-free, SPF flora, SPF mice were given 0.1 mg of ciprofloxacin (Ciproxin; Bayer, Leverkusen, Germany) orally twice a day for three consecutive days. Ciprofloxacin has previously been shown to selectively eliminate SFBs (15).

Four groups of 15 germ-free mice each were housed in germ-free isolators. Based on experiments described earlier (17), two groups of mice were associated with SFBs by placing in their cages fresh faeces derived from mice mono-

associated with SFBs. One group of germ-free mice and one group exposed to SFB containing faeces were inoculated with caecal homogenate prepared from SFB-free, SPF mice; 0.5 ml of homogenate was given orally twice within 48 hours.

All mice received a sterilized, pelleted, commercial diet (SRM-A, Hope Farms BV, Woerden, The Netherlands) and sterilized, demineralized water *ad libitum*.

Microbiological status of mice used

Faecal samples from mice mono-associated with SFBs (n=10) were suspended in saline. Gram-stained smears were prepared and examined microscopically for the presence of SFBs. Faecal samples were also cultured aerobically (for 2 days) and anaerobically (for 5 days) on non-pre-reduced and pre-reduced, enriched blood agar plates, respectively (24). Faecal samples from germ-free mice were examined in an identical manner. Microscopic and cultural examination of faeces from mice mono-associated with SFBs indeed showed that all faecal samples were SFB-positive, and that no bacteria could be cultured *in vitro*. Thus, faeces of these mice was considered suitable for mono-association of germ-free mice with SFBs. Faecal samples of the germ-free mice did neither contain SFBs, nor cultivable bacteria.

Ten SPF, ciprofloxacin-treated and four non-treated mice were killed by cervical dislocation and the caeca with contents removed aseptically, and subsequently pooled per treatment group and homogenized in broth (19). Sheep blood and glycerol were added to concentrations of 7 and 20% (v/v). Caecal homogenates were stored at -80°C until use. Total bacterial counts of caecal homogenates were obtained as described (11). 0.1 ml of tenfold diluted homogenates were spread and incubated in duplicate in different media (Table 1) to determine the concentrations of selected aerobic and anaerobic bacteria. Table 2 shows that ciprofloxacin did not appreciably change the cultivable caecal flora composition. SFBs in mucosal smears of the distal small intestine were ascertained by microscopy (14). In ciprofloxacin-treated SPF mice no SFBs could be detected. Thus, ciprofloxacin indeed selectively eliminated SFBs. Six germ-free mice that were inoculated orally with caecal homogenates from ciprofloxacin-treated mice were killed by cervical dislocation five days after inoculation and also examined for SFBs. All mice were found to be free from SFBs.

Table 1. Culture media and incubation conditions to assess the effect of ciprofloxacin treatment on caecal microflora

Medium	Organisms cultured	Incubation method[e]	Incubation time (days)
Non-selective media			
non pre-reduced, enriched blood agar[a]	aerobes	air	2
pre-reduced, enriched blood agar[a]	anaerobes	anaerobic glove box[a]	5
Selective media			
Mannitol Salt agar[b]	staphylococci	air	2
Kanamycin Aesculin Azide agar base[c]	streptococci		
Levine EMB agar[b]	Enterobacteriaceae		
Rogosa agar[b]	lactobacilli	anaerobic glove box[a]	5
Reinforced Clostridial agar[c]	clostridia		
Bacteroides Bile Esculin agar[d]	Bacteroidaceae		
Brucella Medium base agar[c]	bifidobacteria		
Sabouraud Dextrose agar[c]	yeasts and moulds	air	2

[a] Described elsewhere (5).
[b] Supplier: Difco.
[c] Supplier: Oxoid.
[d] Described elsewhere (8).
[e] Temperature was always 37°C.

Four mice per experimental group were killed by cervical dislocation. Caecal weight including contents was determined. The distal small intestine, caecal contents and faeces were examined for the presence of SFBs as described above. Microscopic and cultural examination of diluted caecal homogenates were carried out for two mice per experimental group as described above. The percentage of

fusiform-shaped bacteria in Gram-stained smears of caecal contents was determined. The experimental mice with SFBs were indeed found to be SFB-positive (Table 3). In the SFB-positive, SPF mice, SFBs were seen only in the ileum. Tables 3 and 4 show that the gut flora of the two experimental groups of SPF mice strongly resembled that of the SPF mice used as starting animals (Table 2). The only difference was the absence of staphylococci in the experimental SPF mice.

Table 2. Caecal flora in ciprofloxacin-treated and non-treated SPF mice

Microbiological parameter	Non-treated mice	Treated mice
	Log_{10} n/g caecum with contents[1]	
Total count	10.7	10.9
Vital aerobic count	5.6	6.3
Vital anaerobic count	10.3	9.4
Staphylococci	4.0	3.7
Streptococci	4.3	5.2
Enterobacteriaceae	3.7	5.3
Lactobacilli	7.3	8.6
Bifidobacteria	n.d.[2]	n.d.
Clostridia	8.3	7.2
Bacteroidaceae	8.1	6.7
Yeasts/moulds	n.d.	n.d.

[1] Presented are mean values for cultures in duplicate (caeca were pooled from all untreated and all treated mice, respectively).
[2] Not detectable.

From 2 or 4 mice per experimental group, samples of the ileum and caecum were used for scanning electron microscopy to further check for the presence of SFBs. Pieces of ileum located about 1 cm proximally to the ileocaecal junction and portions of the middle part of the caecum were dissected, flushed with saline and fixed for 24 h at 20°C in 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer solution (pH 7.4, 320 mOsmol). Tissue samples were processed and

scanning electron micrographs taken as described (21). From the mice mono-associated with SFBs, two and four out of four animals were found to be SFB-positive as based on ileal and caecal samples, respectively. Fig. 1 illustrates SFBs attached to the caecal mucosa. In the other three experimental groups, no SFB-positive animals could be detected.

Table 3. Selected microbiological parameters in the four experimental groups

	Experimental group			
	Germ-free mice	SFB-positive mice	SFB-negative, SPF mice	SFB-positive, SPF mice
Microbiological parameter:				
Caecal weight, g/100 g body weight	9.1 ± 0.7	5.2 ± 0.5	2.7 ± 0.5	2.5 ± 0.4
Caecal fusiforms, number per 100 bacteria	0	0	67 ± 5	77 ± 3
Presence of SFBs, SFB-positive animals/total				
Ileum	0/4	4/4	0/4	2/4
Caecum	0/4	2/4	0/4	0/4
Faeces	0/4	4/4	0/4	0/4
Total count, $\log_{10}$ n/g caecum with contents	n.d.	n.d.	10.9, 11.1	10.7, 10.8
Vital aerobic count, idem	n.d.	n.d.	7.9, 8.0	8.1, 8.2
Vital anaerobic count, idem	n.d.	n.d.	10.8, 10.9	10.5, 10.5

Means ± SD for 4 mice per group or individual values for 2 mice per group; n.d. = not detectable.

Table 4. Caecal flora in experimental SPF mice without or with SFBs

Group of caecal bacteria	SFB-negative, SPF mice	SFB-positive, SPF mice
	Log_{10} n/g caecum with contents	
Staphylococci	n.d.	n.d.
Streptococci	6.8, 7.0	5.4, 5.8
Enterobacteriaceae	6.0, 6.5	6.5, 6.8
Lactobacilli	8.6, 9.0	8.0, 8.1
Bifidobacteria	n.d.	n.d.
Clostridia	9.8, 9.8	8.4, 8.5
Bacteroidaceae	7.0, 7.5	7.9, 8.2
Yeasts/moulds	n.d.	n.d.

Individual values are given for 2 mice per group; n.d. = not detectable.

Lymphoid cells in lamina propria

From two mice per experimental group, the distal half of the small bowel was removed, flushed with sterile saline and slit open longitudinally. A Swiss roll was made and the tissue was fixed, sectioned and stained with conventional histological techniques (P.A.S. and hematoxylin; Goldner, i.e. Jerusalem's modification of Masson's trichrome stain), as described (7). The same was done with whole caeca. Light microscopic examination of the slides was used to assess the presence of lymphoid cells in the lamina propria of the ileum and caecum. The number of lymphoid cells (lymphocytes and/or plasma cells) was scored on a scale from 0 (lymphoid cells essentially absent) to 3 (relatively large number of lymphoid cells). The presence of SFBs was also checked. Based on examination of the slides from caecal samples, the two mice mono-associated with SFBs were indeed SFB-positive. Fig. 2 illustrates an SFB at the caecal mucosa. The other animals were all negative. In slides from ileal samples no SFBs were detected, irrespective of the experimental group. This was probably due to the relatively low degree of SFB colonization in the SFB-positive mice (data not shown).

Five mice per group were killed by carbon dioxide inhalation. Spleen and small intestine were removed. Spleens were placed in Hanks' solution, minced with scissors and squeezed through a nylon gauze filter (100 μm) to obtain a single cell suspension. Viability was always higher than 90% as determined by nygrosin exclusion. Lamina propria cell suspensions were prepared as described in detail (37). Briefly, the intestine was rinsed and the Peyer's patches were excised. Subsequently, the intestine was cut longitudinally, washed in Ca- and Mg-free, balanced salt solution (CMF) and cut into small pieces of 0.5-1 cm. The mucosal tissue strips were incubated for 10-15 min in CMF containing 0.37 mg EDTA and 0.145 mg DTT per ml. After filtration, the debris was further incubated for 75-90 min in RPMI-1640 (Serva, Heidelberg, Germany) containing 5% FCS, 20 mM HEPES and 0.1 mg DNA-ase per ml. The supernatant was collected and squeezed through nylon gauze filters (100 and 50 μm) to provide a single cell suspension. The tissue strips remaining after collagenase digestion were squeezed through nylon gauze filters (200, 100 and 50 μm) to provide a second single cell suspension. Both suspensions were pooled and washed with RPMI-1640. Viability of the isolated cell suspension was about 50%. To avoid bacterial growth, kanamycin was added to all buffer solutions used in the isolation procedures.

The modified protein A plaque-forming cell (PFC) assay (39) was used to count the number of immunoglobulin secreting cells (Ig SC). Plaques were read after incubating the slides for 4 hours at 37°C. Commercially available rabbit anti-mouse IgM and IgA antisera (Nordic, Tilburg, The Netherlands) and ditto IgG antiserum (Miles, Slough, Berks, UK) were used. Guinea pig complement (Behringwerke, Marburg-Lahn, Germany) was absorbed by passage over a Sepharose-bound protein A (Pharmacia, Uppsala, Sweden) column (39). All antisera were selected on the basis of their specificity, which was determined by ELISA techniques performed on microtitre plates coated with purified myeloma IgM, IgG2A or IgA (Litton Bionetics, Charleston, SC, USA) as described (38).

IgA in sera and intestinal secretions

From five mice per group, killed by exposure to carbon dioxide, blood samples were taken by heart puncture. Intestinal secretions were obtained by a scraping technique described elsewhere (35). IgA was quantified in sera and secretions by ELISA in microtitre plates coated with rabbit anti-mouse IgA. Sheep anti-mouse

IgA peroxidase conjugate was purchased from Serotec Ltd, Bicester, UK. Bound conjugate was visualized by adding 3,3',5,5'-tetramethylbenzidin (Boehringer, Mannheim, Germany) and H_2O_2 as substrate. The colouring reaction was stopped after 10 min with 0.5 M H_2SO_4 and the absorbance at 450 nm of each well measured. Results were expressed as the reciprocal dilution that gave an extinction $\geq$ 0.150 above background.

T cell proliferation assay

From two animals per group, killed by cervical dislocation, Peyer's patches, mesenteric lymph nodes and spleen were removed and pooled per group. Single cell suspensions were made by gently teasing out in RPMI-1640 Dutch modification (dm) (Flow Laboratories Inc., McLean, VA, USA). The cells were washed once with RPMI-1640 dm and erythrocytes lysed by incubation in 0.16 M NH_4Cl and 0.17 M Tris-HCl (pH 7.2) for 2 min at 20°C. The cells were washed twice with RPMI-1640 dm and suspended in IMDM supplemented with 2% (v/v) SF1 (Costar, Cambridge, MA, USA) as a serum substitute, 20 mM glutamine, 10 mM pyruvate and 40 μg gentamycin per ml. Adherent cells in the splenic cell suspensions were removed by addition to a culture flask which was placed in a carbon dioxide incubator for 60 min at 37°C. Non-adherent cells were collected by aspiration. The proliferation assay was carried out as described (33), with minor modification. Cell suspensions were incubated in 96-well, round-bottom microtitre plates (Costar) at a density of $5x10^5$/ml and stimulated with concanavalin A (Con A; 1 μg/ml). After 72 h in a carbon dioxide incubator at 37°C, $3.7x10^4$ Bq [^{3}H]thymidine (specific activity, 0.7-1.1x10^8 MBq/mmol; Amersham Nederland BV, The Netherlands) was added per well. After 24 h, the cells were harvested onto glass fibre filter papers (Titertek; Flow Laboratories). The filter papers were placed in Aqualuma (Lumac BV, The Netherlands) and the amount of radioactivity counted (LKB Wallac 1214 Rack-beta). In control experiments, Con A was replaced by phosphate buffered saline. Stimulation indices were calculated as the number of counts in Con A-stimulated cell cultures divided by that in control cultures.

Statistics

Analysis of variance (ANOVA) was used to evaluate differences in measured parameters between the four experimental groups. The effect of SFBs and SPF gut flora were main effects. A priori defined contrasts between the experimental groups with a single variable were evaluated with Student's *t* test. A P value <0.05 was pre-set as criterion of statistical significance.

RESULTS

Lymphoid cells in lamina propria

Fig. 3 shows that the ileum of mice mono-associated with SFBs is enriched in lymphoid cells when compared with germ-free mice. The number of lymphoid cells in the lamina propria of the distal small intestine and the caecum was markedly increased in SFB-positive mice, irrespective of whether or not they had a SPF gut flora (Table 5).

Table 5. Presence of lymphoid cells in lamina propria of ileal and caecal mucosa of mice from the experimental groups

	Experimental group			
Site	Germ-free mice	SFB-positive mice	SFB-negative, SPF mice	SFB-positive, SPF mice
Ileum	0, 0	2, 3	1, 2	3, 3
Caecum	0, 0	3, 3	1, 2	3, 3

Individual results are given for 2 mice per group; score on a scale from 0 (lymphoid cells essentially absent) to 3 (relatively large number of lymphoid cells).

Total numbers of IgM-, IgG- and IgA-secreting cells (sc) in suspensions of spleen and small intestine are shown in Fig. 4. Mice that harboured an intestinal flora had higher numbers of intestinal IgA-sc than did germ-free mice, the highest number being observed in mice mono-associated with SFBs. The stimulatory influence of SFBs on IgA-sc was not seen in the presence of the SPF flora. The total numbers of IgM- and IgG-sc in the intestinal mucosa were lower than that of IgA-sc and did not differ significantly between the groups. The frequencies of Ig-sc in the small intestine are shown in Table 6. As to IgA-sc, the frequencies show the same differences as the total number of cells. Thus, the SFB-induced increase of the number of IgA-sc in the absence of the SPF flora was not caused by a higher

Table 6. Total number of living cells and Ig-secreting cells (Ig-sc) in the small intestine of mice from the experimental groups

	Experimental group					
	Germ-free mice	SFB-positive mice	SFB-negative, SPF mice	SFB-positive, SPF mice	ANOVA	Contrast
Number of living cells ($x10^6$)	225 ± 28	180 ± 28	108 ± 16	131 ± 12	-	-
Number of Ig-sc/10^6 cells						
IgM	29 ± 7	13 ± 5	30 ± 6	16 ± 5	SFB	a,b
IgG	121 ± 35	434 ± 56	426 ± 18	815 ± 48	SFB,SPF	a,b,c,d
IgA	969 ± 164	14326 ± 3329	7480 ± 1785	6974 ± 1089	SFBxSPF	a,c,d

Means ± SEM, n=5. ANOVA significance ($P<0.05$): SFB = effect of SFBs; SPF = effect of SPF gut flora; SFBxSPF = interaction. Contrast significance ($P<0.05$): a = SFB effect in the absence of other bacteria; b = SFB effect in the presence of a SPF gut flora; c = SPF effect in the absence of SFBs; d = SPF effect in the presence of SFBs.

recovery of cells per organ. Table 6 also shows that SFBs lowered the relative number of IgM-sc and raised that of IgG-sc in the small intestine. The type of intestinal flora had no major impact on the number of splenic Ig-sc, but SFBs in the presence of the SPF flora raised the number of IgM- and IgG-sc (Fig. 4).

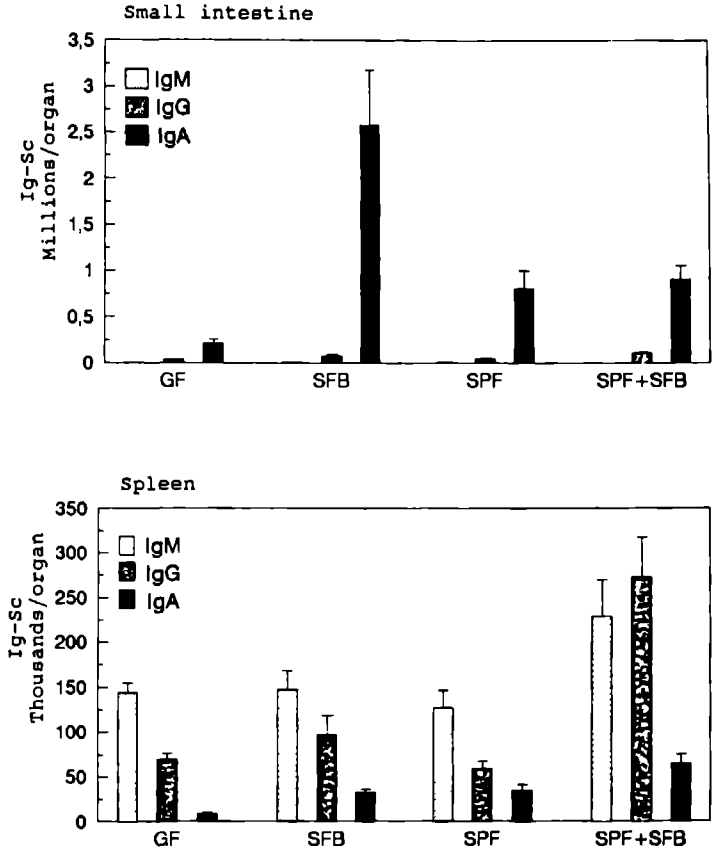

Fig. 4. Total numbers of IgM-, IgG- and IgA-secreting cells (sc) per organ in small intestine and spleen derived from mice of the four experimental groups. Experimental groups: GF = germ-free mice; SFB = SFB-positive mice; SPF = SFB-negative, SPF mice; SPF+SFB = SFB-positive, SPF mice. Results are means ± SEM (bars) for 5 mice per group.

IgA in sera and intestinal secretions

Table 7 shows that mice mono-associated with SFBs versus germ-free mice had significantly higher IgA titres in serum and intestinal secretions. This also held for SPF mice without SFBs versus germ-free mice. In mice with a SPF flora, SFBs raised the amount of IgA in serum, but not that in intestinal secretions.

Table 7. Titres of IgA in serum and intestinal secretions of mice from the experimental groups

	Experimental group					
	Germ-free mice	SFB-positive mice	SFB-negative, SPF mice	SFB-positive, SPF mice	ANOVA	Contrast
Serum IgA	230 (264-200)	1393 (1600-1213)	528 (623-447)	919 (1056-800)	SFB,SPF, SFBxSPF	a,b,c
Secretory IgA	131 (156-112)	696 (800-606)	460 (528-400)	528 (623-447)	SFB, SFBxSPF	a,c

Means and range (between parentheses), n=5. ANOVA significance ($P<0.05$): SFB = effect of SFBs; SPF = effect of SPF gut flora; SFBxSPF = interaction. Contrast significance ($P<0.05$): a = SFB effect in the absence of other bacteria; b = SFB effect in the presence of a SPF gut flora; c = SPF effect in the absence of SFBs; d = SPF effect in the presence of SFBs.

T cell proliferation assay

Cells derived from Peyer's patches, mesenteric lymph nodes and spleen were tested for their proliferative response after incubation with Con A. Table 8 shows that the proliferative response of Peyer's patch cells was negligible and also similar between the groups. SFBs markedly enhanced the proliferative response of

mesenteric lymph node cells, but this was seen only in the absence of the SPF flora. In mesenteric lymph node cells isolated from SFB-free SPF mice, the response was markedly raised when compared to that in cells from germ-free mice. This was also seen for the proliferative response in spleen cells. Both in the absence and presence of the SPF flora, SFBs reduced the proliferative response in spleen cells.

Table 8. *In vitro* concanavalin A-stimulated proliferation of Peyer's patch cells (PPC), mesenteric lymph node cells (MLNC) and spleen cells (SPLC) isolated from mice of the experimental groups

	Experimental group			
Cell type[1]	Germ-free mice	SFB-positive mice	SFB-negative, SPF mice	SFB-positive, SPF mice
	Stimulation index[2]			
PPC	2	3	<1	2
MLNC	1	6338	1560	2026
SPLC	1079	690	2179	228

[1] Cells were isolated from pooled tissues of 2 mice per experimental group; proliferation assays were done in triplicate and means presented.

[2] Proliferative response expressed as stimulation index, i.e. the number of counts in Con A-stimulated cell cultures divided by the number of counts in control cultures.

DISCUSSION

We have examined the effect of SFBs on the immune system of mice, with special reference to the mucosal immune compartment. To ensure that observed effects

could be attributed to SFBs in the intestinal tract, the microbiological status of the animals was carefully monitored with the use of various techniques (Table 3, Fig. 1 and 2). Based on the results, we conclude that differences between germ-free and SFB-positive mice and between SFB-negative, SPF mice and SFB-positive, SPF mice must have been caused solely by SFBs.

Various observations indicate that SFBs strongly stimulate the mucosal immune compartment. SFBs versus no microbial flora produced a rise of the numbers of IgA-sc in the lamina propria of the small intestine, increased the IgA titres in serum and intestinal secretions and enhanced the Con A-induced proliferative response of mesenteric lymph node cells. These findings were supported by increased numbers of lymphoid cells in histological sections of the ileum and caecum of mice mono-associated with SFBs. The SFB-induced increase in serum IgA titres and that in the number of lymphoid cells in lamina propria of ileal and caecal mucosa were also seen in the presence of the SPF flora. All stimulatory effects can be considered rather specific for SFBs because the influence of the SPF flora on these parameters was less pronounced. However, in mice associated with the SPF flora, certain effects of SFBs were diminished or even absent. Possibly, the presence of the SPF flora interferes with intestinal colonization by SFBs, so that certain stimulatory effects of SFBs are diminished. Indeed, microscopic examinations revealed that SFBs were less abundant in mice associated with both the SPF flora and SFBs than in mice mono-associated with SFBs.

Although SFBs clearly stimulated the mucosal immune compartment, the effect on the systemic immune compartment was equivocal. SFBs raised the serum IgA concentration and the number of IgA-sc in spleen. SFBs also raised the number of IgM- and IgG-sc in spleen, provided that the mice had the SPF flora. On the other hand, SFBs systematically reduced the Con A-induced proliferative response of isolated spleen cells. This may be explained by recruitment of reactive cells towards mucosal sites. In these experiments, samples were taken only two weeks after inoculation of mice with gut floras. This indicates that the mucosal immune compartment is stimulated very rapidly.

The steep increase of intestinal IgA-sc as induced by SFBs resembles the sudden increase of IgA-sc in mice after weaning (36). It is well documented that the intestinal bacterial flora can stimulate the mucosal immune compartment (25, 27, 28, 36). Moreover, a complex microbial flora has been reported to raise

concentrations of serum immunoglobulins in chickens, rats and mice (10). However, the relative contribution of the various bacterial species or strains is not known. Moreau *et al.* (28) stated that unknown non-cultivable microorganisms are responsible for the attainment of normal levels of IgA-containing cells in the small intestine. We now postulate that these microorganisms are SFBs. Indeed, SFBs possess the characteristics of the unknown bacteria described by Moreau *et al.* (28), i.e. non-cultivable, heat- and bacitracin-sensitive, absent in mice before weaning and present in high numbers shortly after weaning (8, 20-22).

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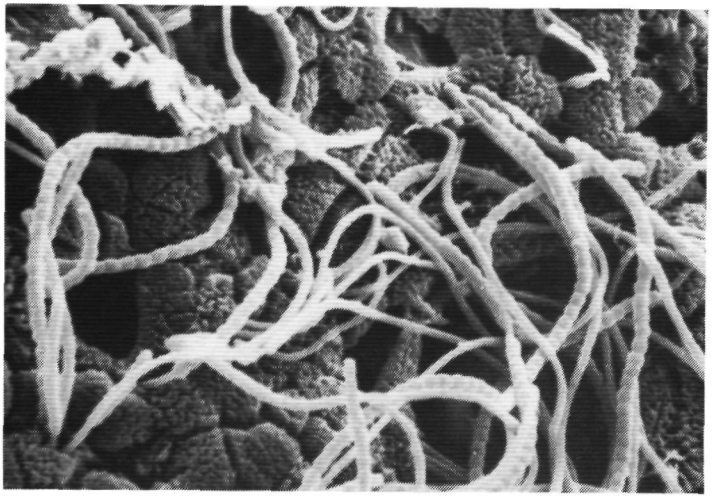

Fig. 1. Scanning electron micrograph of SFBs attached to the caecal mucosa of a mouse mono-associated with SFBs (magnification, 1400x).

Fig. 2. SFB (arrow) in the caecum of a mouse mono-associated with SFBs (Goldner's trichrome stain; magnification, 960x).

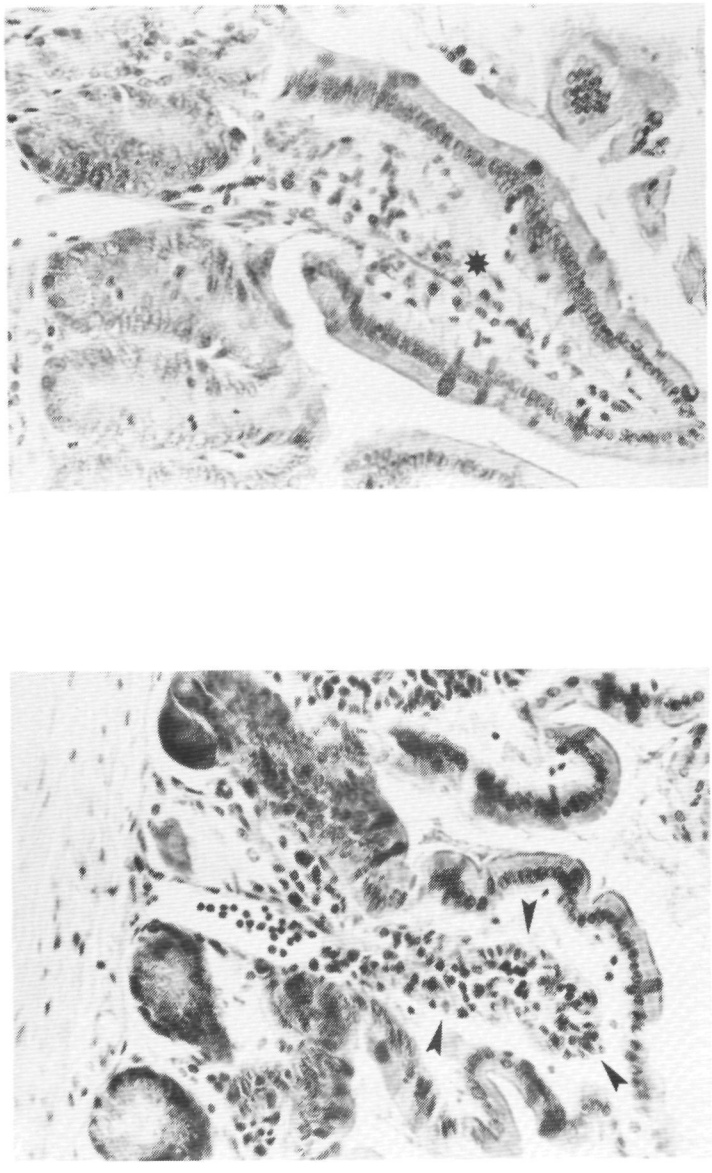

Fig. 3. A. Villus of the ileum of a germ-free mouse (PAS-Hematoxylin staining; magnification, 280x). There is a low cell density in the lamina propria (*); the majority of the cells are fibrocytes with only few lymphoid cells.
B. Villus of the ileum of a mouse mono-associated with SFBs (PAS-Hematoxylin staining; magnification, 280x). There is an increased density in the lamina propria due to lymphoid cells (arrows).

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4.2 Intestinal, segmented, filamentous bacteria and colonization resistance of mice to pathogenic bacteria

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SUMMARY

It has been suggested that segmented, filamentous bacteria (SFBs) in the ileum of mice play a role in host resistance to pathogens. This suggestion was tested by comparing four groups of mice, namely germ-free mice, mice mono-associated with SFBs, SFB-free mice with a specified pathogen-free (SPF) flora, and SFB-positive, SPF mice. Colonization of host tissues was determined after oral administration of viable cells of *Salmonella enteritidis* and *Enterobacter cloacae*. Serum IgA concentrations were measured as well as the *in vitro* proliferative responses of Peyer's patch cells (PPC), mesenteric lymph node cells (MLNC) and spleen cells (SC) to various antigens. The SPF gut flora reduced colonization of host tissues by pathogens, whereas SFBs did not. Mice mono-associated with SFBs had a lower incidence of salmonella-positive livers and spleens than germ-free mice. When compared with the germ-free status, both the SPF flora and SFBs raised the serum IgA concentration. The SPF flora consistently raised the concanavalin A-induced proliferative response of MLNC. In MLNC from SFB-positive, SPF mice, the concanavalin A-induced proliferative response was lower than in SFB-free, SPF mice, but the salmonella antigen-induced response of SC was higher. SFBs raised the salmonella antigen-induced proliferative response of PPC isolated from mice challenged with *Enterobacter cloacae*. It is suggested that activation of MLNC as induced by the SPF flora is related to inhibition of colonization by the selected pathogens, whereas activated lymphocytes with a specific reactivity to these pathogens are relatively poorly present in Peyer's patches.

INTRODUCTION

In the distal small bowel of mice and rats, a characteristic bacterial morphotype occurs, which has not yet been cultured *in vitro*.[2,4,5,19] These microbes, designated as segmented, filamentous bacteria (SFBs), are apathogenic and autochthonous. In the ileum, they are firmly attached to epithelial cells of villi and Peyer's patches.[1,2,4-6] The impact of SFBs on their host is unknown, but it has been suggested that SFBs enhance gastrointestinal colonization resistance to

enteropathogens.[6,12,15,17] The association of SFBs with Peyer's patch epithelium at which they frequently form a dense microbial layer, has raised the hypothesis that SFBs enhance host immunity to enteropathogenic bacteria.[7,9]

The objective of this study was to determine the influence of SFBs on the host resistance to two different enteropathogenic bacteria, namely *Salmonella enteritidis* and *Enterobacter cloacae*. After orogastric challenge of SFB-negative and SFB-positive mice, the pathogens were cultured from the intestine and extra-intestinal tissues. In this way, the effect of SFBs on translocation of the administered pathogens could be determined. In addition, serum IgA titres and the *in vitro* proliferative responses of cells from Peyer's patches, mesenteric lymph nodes and spleen were measured.

MATERIALS AND METHODS

Animals and housing

There were four experimental groups of mice: germ-free mice, mice mono-associated with SFBs, SFB-free mice with a specified pathogen-free (SPF) gut flora, and SFB-positive, SPF mice. Female, germ-free NIH mice (a substrain of Swiss mice), aged 4-5 wks, were obtained from the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands. A group of germ-free mice was mono-associated with SFBs by placing in their cages fresh faeces derived from Swiss mice mono-associated with SFBs, which had been produced and were housed as described.[10] Part of the germ-free animals and part of the mice mono-associated with SFBs were associated with gut bacteria from SPF Swiss mice bred in-house. Thus, these mice were inoculated with caecal homogenate prepared from the SPF mice; 0.5 ml of homogenate was given orally twice within 48 h. The SPF microflora has been described.[13] To produce mice with an SFB-free, SPF flora, SPF mice were given 0.1 mg of ciprofloxacin (Ciproxin^{R}, Bayer, Leverkusen, Germany) orally twice a day for three consecutive days. Ciprofloxacin has previously been shown to selectively eliminate SFBs.[11] The germ-free mice were verified to have a gut free of bacteria, and the other groups were found to have gut floras as would be expected (results not shown). All mice were housed in germ-free isolators as described.[10] They all received a sterilized,

pelleted, commercial diet (SRM-A, Hope Farms BV, Woerden, The Netherlands) and sterilized, demineralized water *ad libitum*.

Challenge with Salmonella enteritidis

In the first experiment, two weeks after formation of the experimental groups, all animals of the four groups (n=8/group) were challenged with a strain of *Salmonella enteritidis*, which was a clinical isolate (strain Van Hulst, phage type 1) kindly donated by Dr. E. Goren (Poultry Health Institute, Doorn, The Netherlands). A pure culture of this strain was stored at -80°C in BHI broth (Difco) containing 7% (v/v) sheep blood and 20% glycerol. Before use, the culture was streaked onto Blood Agar Base (Oxoid) supplemented with 5% sheep blood, and incubated overnight at 37°C. Then, colonies were inoculated into 1 ml of BHI broth (Difco) and incubated for 16-18 h at 37°C. Each animal was challenged with *S. enteritidis* by orogastric inoculation of 0.5 ml broth containing $1x10^6$ viable organisms. Challenge dose and the period after challenge until measurements were based on pilot experiments. Part of the mice was examined at 24 h post inoculationem (p.i.) (n=6/group) and at 2 wks p.i. (n=2/group). Individual faecal samples were collected and blood samples were taken by orbital puncture under ether anaesthesia. The anaesthetized animals were killed by cervical dislocation.

Challenge with Enterobacter cloacae

In the second experiment, two weeks after formation of the experimental groups, all animals of the four groups (n=8/group) were challenged with a human strain (AXO) of *Enterobacter cloacae* which was kindly provided by Dr. E.J. Vollaard (Department of Pharmacy, Canisius-Wilhelmina Hospital, Nijmegen, The Netherlands). A pure culture of this strain was stored and subcultured as described above for salmonella. Each animal was inoculated orogastrically with 0.5 ml of broth containing $1x10^9$ viable cells of *E. cloacae*. At 16 h p.i. (n=6/group) and 1 wk p.i. (n=2/group) faecal and blood samples were collected and the animals killed as described above.

SFBs in faeces and small bowel

From individual faeces, Gram-stained smears were prepared and examined microscopically for the presence of SFBs; 100 fields per smear were examined at a magnification of 1000x. The small intestine was aseptically removed, and a portion with length of 1 cm, located adjacently to the ileocaecal junction, was isolated. This piece was cut longitudinally, and contents were removed carefully with a pair of tweezers. Then, the ileal mucosa was vigorously rubbed onto a microscopic slide. The mucosal smear was fixed by dry heat, Gram-stained and examined microscopically as described above for faecal smears.

Colonization by salmonella

All procedures were performed aseptically. Contents of the caecum and distal half of the small bowel were squeezed out and collected in a sterile tube. Then, the gut sections were opened longitudinally and washed 5 times in sterile saline. Intestinal contents and gut tissues were weighed, homogenized in sterile saline and serially diluted in tenfold steps. This was also done for liver and spleen. From appropriate dilutions, 10 μl was plated onto Brilliant Green agar (Oxoid) for salmonella counts. Bacterial counts were expressed as $\log_{10}$ CFU per gram wet weight of gut contents or tissue. If salmonella was not detected, the theoretical maximum value minus 1 was used to calculate group means.

Colonization by enterobacter

Homogenates of small bowel and caecal contents, small bowel, caecum, mesenteric lymph nodes, liver and spleen were prepared as described above, and 10 μl of diluted homogenates was plated onto Levine EMB agar (Difco) containing 16 μg/ml cefotaxime. Bacterial counts were determined.

Serum IgA titre

IgA in serum was quantified by ELISA in microtitre plates coated with rabbit anti-mouse IgA. Sheep anti-mouse IgA peroxidase conjugate was obtained from Serotec Ltd, Bicester, UK. Bound conjugate was visualized by adding 3,3',5,5'-tetramethylbenzidin (Boehringer, Mannheim, Germany) and H_2O_2 as substrate; the

reaction was stopped after 10 min by addition of 0.5 M H_2SO_4 and the absorbance measured at 450 nm. Results are expressed as the reciprocal dilution that gave an extinction $\geq$ 0.150 above background.

T cell proliferation assay

Peyer's patches of the small intestine, mesenteric lymph nodes and part of the spleen were sampled aseptically and pooled per experimental group. Single cell suspensions were made by gently teasing out tissues in RPMI-1640 dutch modification (dm) (Flow Laboratories Inc., McLean, VA, USA). The cells were washed once with RPMI-1640 dm and erythrocytes lysed by exposure to 0.16 M NH_4Cl and 0.17 M Tris-HCl (pH 7.2) for 2 min at 20°C. The cells were washed twice with RPMI-1640 dm and suspended in IMDM supplemented with 2% (v/v) serum substitute SF1 (Costar, Cambridge, MA, USA), 20 mM glutamine, 10 mM pyruvate and 40 μg gentamycin per ml. Adherent cells in the splenic cell suspensions were removed by incubation for 60 min at 37°C in a culture flask which was placed in a carbon dioxide incubator. Non-adherent cells were collected by aspiration.

Bacterial antigens to be used in the proliferation assay were prepared as follows. SFBs were derived from Swiss mice mono-associated with SFBs.[10] Six mice were killed by cervical dislocation and the small intestines removed aseptically. Intestinal contents were gently removed with a pair of tweezers and the intestines with attached SFBs were pooled (0.6 g, wet weight) and subsequently homogenized in 4.5 ml of phosphate buffered (pH 7.4) saline (PBS). Microscopic examination of homogenate indicated an SFB concentration of 10^6 per ml. Control antigen was derived from germ-free Swiss mice following the same procedure. Bacteria from an overnight culture of *S. enteritidis* in BHI broth (Difco) were washed, and brought to a concentration of 10^9 bacteria per ml. The bacterial suspension was incubated for 1 h at 56°C, and then sonicated for 2 min on ice with amplitude of 16 μm (MSE Soniprep 150). Similarly, antigens were prepared from *E. cloacae*, a mouse-derived, neomycin-resistant strain of *Escherichia coli* (O90K-) and a human, norfloxacin-resistant strain of *Klebsiella pneumoniae* (strain Kooiman). The antigens were stored at -20°C. Before use in the proliferation assay, the antigens were thawed and filtered using a 0.45 μm-filter (Schleicher and Schuell, Dassel, Germany). Each antigen was diluted with RPMI-1640 dm.

The proliferation assay was carried out as described[20], with minor modifications. Cell suspensions were incubated in 96-well round-bottom microtitre plates (Costar) at a density of $5x10^5$/ml and stimulated with either concanavalin A (Con A; 1 μg/ml), 25-fold diluted SFB antigen, 25-fold diluted control, germ-free antigen or 125-fold diluted antigen from the various bacterial strains. After 72 h in a carbon dioxide incubator at 37°C, $3.7x10^4$ Bq [^{3}H]thymidine (specific activity 0.7-1.1x10^8 MBq/mmol; Amersham Nederland BV, The Netherlands) was added per well. After another 24 h, the cells were harvested onto glass fibre filter papers (Titertek; Flow Laboratories Inc.). The filter papers were placed into Aqualuma scintillation fluid (Lumac BV, The Netherlands) and the radioactivity counted (LKB Wallac 1214 Rack-beta). Stimulation indices were calculated as the number of counts in cell cultures incubated with mitogen or antigen divided by the number of counts in cultures incubated with phosphate buffered saline.

RESULTS

Table 1 shows the occurrence of SFBs in faeces and small bowel in both experiments. SFBs were found in mice mono-associated with SFBs and in mice with an SPF flora plus SFBs. As expected, germ-free and SPF animals did not have SFBs. In the challenge experiment with *S. enteritidis*, SFB colonization was less dense than in the challenge experiment with *E. cloacae*.

Salmonella counts were higher at 2 wks than at 24 h after challenge (Table 2). Counts in liver and spleen were generally lower than in intestinal samples. In the three mice that died prematurely, salmonella was cultured from liver and spleen (data not shown). The SPF flora versus absence of flora or the SPF flora plus SFBs versus SFBs only systematically lowered salmonella counts at 24 h p.i. (Table 2). SFBs either in the absence or presence of the SPF flora did not influence colonization by salmonella. However, the liver and spleen of mice mono-associated with SFBs tended to have less salmonellas than germ-free mice. More importantly, the number of mice with detectable salmonella in liver and spleen was systematically reduced by SFBs.

Intestinal organs were more heavily colonized by enterobacter than extra-intestinal organs after the challenge (Table 3). The numbers of enterobacter in

organs cultured 16 h and 1 wk p.i. were similar. Except for mesenteric lymph nodes at 1 wk p.i. and liver and spleen at 16 h p.i., mice with an SPF flora had lower numbers of enterobacter than mice without this flora. SFBs did not influence colonization by enterobacter.

Table 4 shows that enterobacter-infected mice with SFBs had higher serum IgA titres than those without SFBs. Such an effect of SFBs was seen in mice challenged with salmonella only in the absence of the SPF flora.

Table 1. Occurrence of SFBs in faeces and small bowel of mice from the experimental groups

		Experimental group[1]			
		GF	SFBs	SPF	SPF+SFBs
		Number of positive animals/total			
Challenge: *S. enteritidis*					
Faeces	24 h p.i.	0/6	0/6	0/6	0/6
	2 wks p.i.	n.d.[2]	0/1[3]	0/2	0/2
Small bowel	24 h p.i.	0/6	4/6	0/6	0/6
	2 wks p.i.	n.d.[2]	0/1[3]	0/2	0/2
Challenge: *E. cloacae*					
Faeces	16 h p.i.	0/6	6/6	0/6	2/6
	1 wk p.i.	0/2	2/2	0/2	0/2
Small bowel	16 h p.i.	0/6	6/6	0/6	5/6
	1 wk p.i.	0/2	2/2	0/2	0/2

[1] GF = germ-free mice; SFBs = mice mono-associated with SFBs; SPF = mice with SFB-free, SPF gut flora; SPF+SFBs = mice with SPF gut flora plus SFBs.
[2] N.d. = not done because the two mice died within 5 days p.i.
[3] One mouse died at 12 days p.i.

Table 2. Colonization by *Salmonella enteritidis* of experimental mice challenged with this bacterium

		Experimental group[1]			
		GF	SFBs	SPF	SPF+SFBs
		Log_{10} CFU/g (positive animals/total)			
Small bowel contents	24 h p.i.	6.2 ± 0.5 (6/6)	5.9 ± 0.3 (6/6)	3.6 ± 0.8 (4/6)	3.1 ± 0.4 (3/6)
	2 wks p.i.	n.d.[2]	7.9[3]	5.9, 6.0	7.2, 6.5
Small bowel	24 h p.i.	4.8 ± 0.2 (6/6)	4.9 ± 0.2 (6/6)	2.3 ± 0.2 (1/6)	2.3 ± 0.2 (1/6)
	2 wks p.i.	n.d.	7.4	5.7, 4.8	5.8, 5.3
Caecal contents	24 h p.i.	9.0 ± 0.2 (6/6)	8.8 ± 0.2 (6/6)	5.4 ± 0.8 (5/6)	6.2 ± 1.0 (5/6)
	2 wks p.i.	n.d.	8.5	7.9, 7.8	8.5, 9.0
Caecum	24 h p.i.	7.2 ± 0.5 (6/6)	7.7 ± 0.2 (6/6)	3.9 ± 0.4 (5/6)	4.3 ± 0.6 (5/6)
	2 wks p.i.	n.d.	8.0	7.1, 5.7	7.5, 7.1
Liver	24 h p.i.	2.9 ± 0.4 (4/6)	2.2 ± 0.3 (1/6)	1.7±0.04 (0/6)	1.8±0.04 (0/6)
	2 wks p.i.	n.d.	6.2	4.8, 4.7	5.2, 4.8
Spleen	24 h p.i.	3.3 ± 0.5 (4/6)	2.3 ± 0.2 (1/6)	2.2±0.04 (0/6)	2.1±0.04 (0/6)
	2 wks p.i.	n.d.	6.7	5.6, 5.2	5.5, 5.3

Means ± SE or individual values are given.

[1-3] See legend to Table 1.

Table 3. Colonization by *Enterobacter cloacae* of experimental mice challenged with this bacterium

		Experimental group[1]			
		GF	SFBs	SPF	SPF+SFBs
		Log_{10} CFU/g (positive animals/total)			
Small bowel contents	16 h p.i.	5.7 ± 0.3 (6/6)	6.4 ± 0.4 (6/6)	4.5 ± 0.6 (6/6)	4.1 ± 0.3 (6/6)
	1 wk p.i.	7.3, 6.1 (2/2)	6.7, 7.1 (2/2)	5.7, 6.4 (2/2)	2.1, 7.2 (1/2)
Small bowel	16 h p.i.	5.1, 0.2 (6/6)	5.2, 0.3 (6/6)	2.4, 0.2 (2/6)	2.2, 0.1 (1/6)
Caecal contents	16 h p.i.	9.0±0.04 (6/6)	9.2±0.08 (6/6)	6.9 ± 0.2 (6/6)	6.5±0.08 (6/6)
	1 wk p.i.	8.4, 8.3 (2/2)	8.1, 8.2 (2/2)	6.7, 6.8 (2/2)	2.0, 6.9 (1/2)
Caecum	16 h p.i.	6.9±0.04 (6/6)	6.8 ± 0.2 (6/6)	4.4 ± 0.2 (6/6)	3.8 ± 0.1 (6/6)
Mesenteric lymph nodes	16 h p.i.	4.9 ± 0.4 (6/6)	5.1 ± 0.5 (6/6)	2.0±0.02 (0/6)	2.0±0.02 (0/6)
	1 wk p.i.	2.7, 2.5 (0/2)	2.8, 2.5 (0/2)	2.5, 2.7 (0/2)	2.7, 2.5 (0/2)
Liver	16 h p.i.	1.9 ± 0.3 (1/6)	2.1 ± 0.3 (1/6)	1.6 ± 0.0 (0/6)	1.7±0.02 (0/6)
Spleen	16 h p.i.	2.2 ± 0.1 (1/6)	2.5 ± 0.3 (1/6)	2.0±0.03 (0/6)	2.3 ± 0.2 (0/6)

Means ± SE or individual values are given.

[1] See legend to Table 1.

Table 4. Serum IgA titres in mice from the experimental groups

	Experimental group[1]			
	GF	SFBs	SPF	SPF+SFBs
	IgA titre (reciprocal dilution factor)			
Challenge: *S. enteritidis*				
24 h p.i. (n=6/group)	<50	111 (45-274)	223 (158-294)	223 (111-446)
2 wks p.i.	n.d.[2]	400[3]	400, 400	100, 50
Challenge: *E. cloacae*				
16 h p.i. (n=6/group)	<50	218 (79-630)	154 (69-362)	259 (97-676)
1 wk p.i.	<50, <50	200, 200	50, <50	50, 400

Means and range (in parentheses) or individual values are given.
[1-3] See legend to Table 1.

The *in vitro* proliferative response of Peyer's patch cells (PPC) from mice inoculated with salmonella after incubation with Con A was absent (Table 5). However, mesenteric lymph node cells (MLNC) from SFB-free, SPF mice and SFB-positive, SPF mice did respond to Con A, the response being greater in the SFB-free, SPF mice. A similar pattern was seen concerning the response of spleen cells (SC). None of the cells tested were stimulated by germ-free or SFB antigen. *Salmonella enteritidis* antigen elicited a proliferative response by MLNC and SC. In the two cell types, the response was somewhat stimulated by SFBs, i.e. cells from SFB-positive, SPF mice had a slightly greater response than those from SPF mice.

After challenge of mice with enterobacter, isolated cells cultured in phosphate buffered saline proliferated much stronger than did these cells after challenge of the donor mice with salmonella (not shown). Con A-induced stimulation of

Table 5. *In vitro* proliferative response of cells isolated from the experimental mice at 2 weeks after challenge with *Salmonella enteritidis*

		Experimental group[1]		
		SFBs	SPF	SPF+SFBs
Stimulus	Cells[2]	Stimulation index		
Concanavalin A	PPC	1	1	1
	MLNC	1	186	47
	SC	3	17	11
Germ-free antigen	PPC	1	2	1
	MLNC	1	1	1
	SC	1	1	1
SFB antigen	PPC	1	1	1
	MLNC	1	1	1
	SC	1	1	1
S. enteritidis antigen	PPC	1	2	1
	MLNC	1	20	39
	SC	13	16	31

Means for three cultures of cells from tissues pooled per group (SFBs, n=1; SPF, n=2; SPF+SFBs, n=2).

[1] See legend to Table 1.

[2] PPC = Peyer's patch cells; MLNC = mesenteric lymph node cells; SC = spleen cells.

proliferation of PPC was enhanced by the SPF flora (Table 6). SFBs versus a germ-free state also tended to stimulate this proliferative response, but in the presence of a SPF flora, SFBs diminished the response. In SPF mice, the presence of SFBs also slightly reduced the Con A-induced response of MLNC, but had an opposite effect in SC. In mice mono-associated with SFBs versus germ-free mice, the Con A-mediated response of MLNC was not affected, whereas that of SC was somewhat enhanced. The responses of the three cell types to SFB antigen were absent. The other antigens, except for salmonella antigen, elicited negligible proliferative responses. The salmonella-induced proliferative response of PPC was

systematically elevated by SFBs. The SPF flora tended to diminish the salmonella-induced response by MLNC.

DISCUSSION

The bacterial strains used to challenge the experimental mice were selected on the basis of their pathogenicity and site of attachment and invasion. Peyer's patches in the murine ileum appear to be intimately involved in the host's response to salmonella.[3,8,14] *S. enteritidis* has invasive properties[3] and attaches to the epithelium of ileal villi and Peyer's patches,[6] which are the ecological niches of SFBs. Thus, *S. enteritidis* seemed to be a suitable pathogen to test the host-protecting effect of SFBs, if any. Based on preliminary, unpublished studies with germ-free and SPF Swiss mice, we selected a strain of *E. cloacae* for the second infection experiment. This pathogen, which affects the human small intestine,[18] was found to be less invasive than *S. enteritidis* (unpublished observations). *S. enteritidis* infection was associated with three deaths within 12 days out of 32 mice. *E. cloacae* infection did not cause mortality within 1 wk.

In MLNC, the concanavalin A-induced response was consistently elevated by the SPF flora, when compared with SFBs as the sole gut bacteria. However, the addition of SFBs to the SPF flora reduced this response in both experiments. In contrast, in SFB-positive, SPF mice the salmonella antigen-induced proliferative response of SC was systematically higher than in SFB-negative, SPF mice. This could point to a selective enhancement by SFBs of cell-mediated immunity to salmonella. In the challenge experiment with *E. cloacae*, the salmonella antigen-induced proliferative response of PPC was consistently raised by SFBs. It is interesting to note that the gut flora has been suggested to prime Peyer's patch T cells involved in the resistance to salmonella.[16] Only the translocation of *S. enteritidis* to liver and spleen was inhibited by SFBs. It could be suggested that the proliferative response of MLNC is a more representative parameter of the reactivity of the gut-associated lymphoid tissue to salmonella and enterobacter than the proliferative response of PPC.

Table 6. *In vitro* proliferative response of cells isolated from the experimental mice at 1 week after challenge with *Enterobacter cloacae*

		Experimental group[1]			
		GF	SFBs	SPF	SPF+SFBs
Stimulus	Cells[2]	Stimulation index			
Concanavalin A	PPC	1	14	224	41
	MLNC	34	28	60	39
	SC	23	32	11	24
SFB antigen	PPC	1	1	1	1
	MLNC	1	1	1	1
	SC	1	1	1	1
S. enteritidis antigen	PPC	1	9	1	8
	MLNC	14	19	10	9
	SC	4	5	4	7
E. cloacae antigen	PPC	1	3	1	2
	MLNC	5	4	3	2
	SC	2	2	1	2
E. coli antigen	PPC	1	1	1	1
	MLNC	3	4	1	1
	SC	1	1	1	1
K. pneumoniae antigen	PPC	1	1	1	1
	MLNC	2	3	2	2
	SC	2	2	1	1

Means for three cell cultures of cells from tissues pooled per group (n=2 for all groups).

[1] See legend to Table 1.

[2] See legend to Table 5.

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Chapter 5

General discussion

The aim of this thesis was to characterize SFBs in mice and to investigate whether they can contribute to the resistance of mice to intestinal infections.

SFBs in the ileum of mice have been studied intensively and described *in situ* with the use of scanning and transmission electron microscopy. Despite many attempts, they have never been cultured *in vitro*, which is probably due to their obligate attachment to intestinal epithelial cells. Knowledge of determinants of the variation in SFB colonization could provide clues as to the significance of SFBs. Thus, the aim of the experiments described in Chapters 2.1 to 2.7, was to evaluate the contribution of selected host and external factors to this variation. The results indicate that genotype, age and social hierarchy are determinants of SFB colonization. In addition, diet composition and antimicrobial drugs can affect colonization of the ileum by SFBs. In contrast, prevention of coprophagy does not reduce SFB appearance, which may imply that a constant ingestion of SFBs is not crucial for the maintenance of SFB density in the ileum. The data thus obtained may also contribute to the design of *in vivo* studies on SFBs with improved reproducibility. This is of great importance for the progress in research on SFBs.

Methods to isolate SFBs from the ileum of mice are described in Chapter 3.1. SFBs seem to produce spore-like bodies, and this characteristic was used to separate SFBs from non-spore-forming bacteria. When combined with dilution techniques, this enabled the production of a pure suspension of SFBs. This was given to germ-free mice, which induced colonization of the gut by SFBs only (Chapter 3.2). The *in vivo* monoculture thus obtained opens the way to the determination of functional characteristics of SFBs, which was hitherto impossible. The role of SFBs, if any, in a variety of intestinal, metabolic activities such as carbohydrate fermentation, production of short-chain fatty acids, steroid hormone metabolism, deconjugation of bile acids, degradation of intestinal mucin, metabolism of carcinogenic substances, etc. (1,2) can now be studied. These microbial activities, which are reflected by faeces composition, are designated as 'microflora-associated characteristics' (MACs), whereas the corresponding parameters in faeces of germ-free animals are called 'germ-free animal characteristics' (GACs) (4). MACs in mice mono-associated with SFBs may be compared with GACs in germ-free mice in order to determine the possible physiological significance of SFBs in the murine ileum. Of special interest is the possible production by SFBs of short-chain fatty acids or other pathogen-inhibiting

products that might improve CR effects. The first functional characteristics of SFBs that have been examined and described in this thesis, are effects on the immune system and gastrointestinal CR of the mouse.

Chapters 4.1 and 4.2 describe experiments in which the effects of SFBs on the immune system of the mouse and on CR to enteropathogenic bacteria, namely *Salmonella enteritidis* and *Enterobacter cloacae*, were determined. SFBs in mono-associated mice induced a strong mucosal immune response as indicated by raised numbers of IgA-secreting plasma cells in the lamina propria of the ileum and raised IgA concentrations in intestinal secretions. An SFB-free, SPF mouse-derived microflora induced a lower response than SFBs. This is a surprising finding, which may stimulate further research on immunological effects of SFBs and their significance for enteric immunity. Because of the synchronism in weanling mice of the sudden appearance of SFBs and the increased activity of the immune system (3,5), it is attractive to speculate that SFBs play a role in the development of the immune system. SFBs did not affect CR to *S. enteritidis* and *E. cloacae*. To further settle this point, modifications of the challenge experiments with regard to challenge strains, challenge doses, frequency and time of examination of the colonization by pathogens (longitudinal instead of transversal studies), etc., should be carried out.

SFBs are interesting microorganisms that may have an impact on gut function in mice and possibly also in other host species, including man. Therefore, further research on the functional characteristics of these bacteria is needed. For production of SFBs without the use of mice, but also for studies with SFBs not contaminated by host cells or host-derived substances, an *in vitro* cultivation technique will be necessary.

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Summary

In addition to the immune system *per se*, autochthonous, non-pathogenic bacteria in the gut determine host resistance to infections with enteropathogenic bacteria. These gut inhabitants do not only inhibit pathogens by direct interference ('colonization resistance', CR), but also by enhancing effects on immunological mechanisms of the host. This thesis describes various aspects of the segmented, filamentous bacterium (SFB) from the ileum of mice. Host-associated factors, namely genotype, age and social hierarchy, and external factors, namely diet composition and antimicrobial drugs, affect colonization of the ileum of mice by SFBs. Standardization of these factors might reduce unwanted variation in SFB colonization *in vivo* and improve the reproducibility of results.

Other experiments were carried out to isolate SFBs from the ileum of mice. By chemical treatment and dilution of intestinal homogenates from specified pathogen-free (SPF) mice, it was possible to obtain suspensions with viable SFBs and without other living bacteria. An *in vivo* monoculture of SFBs was produced by inoculating germ-free mice with these suspensions. The possibilities thus obtained to study functional aspects of SFBs are discussed.

In a first attempt to functionally characterize SFBs, immunological parameters were determined in mono-associated mice. In addition, the influence of SFBs on colonization and translocation by *Salmonella enteritidis* and *Enterobacter cloacae* was studied. SFBs induced a strong mucosal immune response, particularly indicated by a raised production of IgA in the small intestine. Only minor effects of SFBs on CR to *S. enteritidis* and *E. cloacae* were demonstrated. SFBs did not affect CR to *S. enteritidis* and *E. cloacae*. These observations are discussed.

Samenvatting

Een studie van gesegmenteerde, filamenteuze bacteriën in het ileum van de muis

Naast het immuunsysteem is de darmflora, bestaande uit talloze autochtone, apathogene bacteriële species, van belang voor de weerstand van mens en dier tegen infekties met enteropathogene bacteriën. De beschermende werking van deze apathogene darmflora wordt verklaard door direkte interferentie met pathogenen ("kolonisatieresistentie", KR) en door stimulatie van het immuunsysteem.

Dit proefschrift beschrijft verschillende aspekten van de autochtone, apathogene, gesegmenteerde, filamenteuze bacterie (SFB) in het ileum van de muis.

Gastheerfaktoren zoals genotype, leeftijd en positie in de sociale rangorde, en externe faktoren zoals voedersamenstelling en antibiotica kunnen het kolonisatieniveau van SFB's sterk beïnvloeden. Standaardisatie van deze faktoren kan ongewenste variatie in SFB-kolonisatie reduceren en de reproduceerbaarheid van resultaten verhogen.

Technieken worden beschreven voor het isoleren en vervolgens in kiemvrije muizen tot reinkweek brengen van SFB's. Deze technieken bestaan uit een combinatie van chemische behandeling en verdunning van darmhomogenaten van "specified pathogen-free" muizen. Met de aldus verkregen *in vivo* monocultuur van SFB's in muizen is nu voor het eerst een model beschikbaar, om SFB's nader te bestuderen. Een aantal mogelijkheden wordt genoemd.

In een eerste poging om de funktionele betekenis van SFB's te onderzoeken, werden immunologische reakties bepaald bij muizen mono-geassocieerd met SFB's. Bovendien werd de invloed van SFB's op de KR en de translocatie door orogastrisch toegediende pathogenen (*Salmonella enteritidis* en *Enterobacter cloacae*) bestudeerd. SFB's bleken een sterke mucosale immuunreaktie te induceren, hetgeen met name werd gekenmerkt door een hoge IgA-produktie in de dunne darm. Daarentegen werd geen effekt van SFB's op de KR tegen *S. enteritidis* en *E. cloacae* aangetoond.

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11. **Klaasen HLBM, Peters B, JP Koopman, FGJ Poelma, ME van den Brink, MH Bakker and AC Beynen.** Different degree of ileal colonization by segmented, filamentous bacteria in two strains of mice, submitted.

12. **Klaasen HLBM JP Koopman, FGJ Poelma, ME van den Brink, MH Bakker and AC Beynen.** Intestinal, segmented, filamentous bacteria and colonisation resistance of mice to pathogenic bacteria, submitted.

13. **Klaasen HLBM, JP Koopman, ME van den Brink, MH Bakker and AC Beynen.** Influence of a natural-ingredient diet containing *Phaseolus vulgaris* on the colonization by segmented, filamentous bacteria of the small bowel of mice, submitted.

14. **Klaasen HLBM, PJ van der Heijden, W Stok, FGJ Poelma, JP Koopman, ME van den Brink, MH Bakker, WMC Eling and AC Beynen.** Apathogenic, intestinal, segmented, filamentous bacteria stimulate the mucosal immune system of mice, submitted.

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Curriculum vitae

Eric Klaasen werd geboren te Vroomshoop (Overijsel) op 19 augustus 1958. In 1976 behaalde hij het diploma gymnasium ß aan het Gemeentelijk Lyceum te Eindhoven. In hetzelfde jaar maakte hij een aanvang met de studie diergeneeskunde aan de Rijksuniversiteit te Utrecht die echter twee maal voor een langere periode onderbroken werd; in het eerste geval door zijn tijdelijke toewijding aan jeugdvormingswerk, in het tweede geval door de vervulling van zijn militaire dienstplicht als Officier Civiele Vakopleiding.

In de afstudeerfase van de diergeneeskundestudie werd na een literatuuronderzoek een referaat geschreven, met als onderwerp: "Celgebonden mechanismen bij de IgA-afhankelijke afweer tegen bacteriële enteropathogenen i.h.a. en salmonellae i.h.b. Een fundament voor orale vaccins?". Het schrijven van dit referaat werd begeleid vanuit de Vakgroep Infektieziekten en Immunologie, door dr. J.L. Cornelisse en dr. E.G. Hartman (beiden dierenarts-bacterioloog) en dr. E.J. Hensen en dr. H.K. Parmentier (beiden immunoloog). Voorts werd in deze fase gekozen voor de richting landbouwhuisdieren, waarna het dierenartsdiploma werd behaald in augustus 1987.

Van februari 1988 tot oktober 1991 was de auteur werkzaam op het Centraal Dierenlaboratorium (CDL) aan de Katholieke Universiteit Nijmegen. Hier vervulde hij twee funkties, namelijk die van dierenarts belast met de gezondheidszorg van de proefdieren van het CDL en die van wetenschappelijk onderzoeker. In deze laatste funktie voerde hij het promotie-onderzoek uit dat in dit proefschrift wordt beschreven en dat werd geleid door dr. J.P. Koopman (CDL) en prof. dr. A.C. Beynen (Vakgroep Humane Voeding, Landbouwuniversiteit Wageningen, Vakgroep Proefdierkunde, Rijksuniversiteit Utrecht en Faculteit Geneeskunde, Universiteit van Djakarta, Indonesië).

Sinds 1 oktober 1991 is Eric Klaasen als wetenschappelijk medewerker verbonden aan de Afdeling Microbiologie van het Nederlands Instituut voor Zuivelonderzoek (NIZO) te Ede.